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(54) **ISOLEMENT DE LIGANDS PEPTIDIQUES A SPECIFICITE TISSULAIRE ET UTILISATION DE CES DERNIERS
POUR CIBLER DES SUBSTANCES PHARMACEUTIQUES SUR DES ORGANES**
(54) **ISOLATION OF TISSUE SPECIFIC PEPTIDE LIGANDS AND THEIR USE FOR TARGETING
PHARMACEUTICALS TO ORGANS**

(57)

The subject invention provides novel peptides and the use of these peptides in the treatment of various diseases and conditions. The novel peptides specifically bind to undetermined and determined targets in various organs and in lymphocytes. The subject invention also provides a method for the identification of a peptide by applying peptide library methodology ex vivo to perfused organs.



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(54) **ISOLEMENT DE LIGANDS PEPTIDIQUES A SPECIFICITE
TISSULAIRE ET UTILISATION DE CES DERNIERS POUR
CIBLER DES SUBSTANCES PHARMACEUTIQUES SUR DES
ORGANES**
(54) **ISOLATION OF TISSUE SPECIFIC PEPTIDE LIGANDS AND
THEIR USE FOR TARGETING PHARMACEUTICALS TO
ORGANS**

(57) Cette invention concerne de nouveaux peptides et leur utilisation dans le traitement de diverses maladies et pathologies. Ces nouveaux peptides se lient spécifiquement à des cibles déterminées ou non, situées dans divers organes et dans des lymphocytes. Cette invention concerne également un procédé d'identification d'un peptide qui consiste à appliquer ex vivo une méthodologie de banque de peptides à des organes perfusés.

(57) The subject invention provides novel peptides and the use of these peptides in the treatment of various diseases and conditions. The novel peptides specifically bind to undetermined and determined targets in various organs and in lymphocytes. The subject invention also provides a method for the identification of a peptide by applying peptide library methodology ex vivo to perfused organs.



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(54) Title: ISOLATION OF TISSUE SPECIFIC PEPTIDE LIGANDS AND THEIR USE FOR TARGETING PHARMACEUTICALS TO ORGANS (57) Abstract <p>The subject invention provides novel peptides and the use of these peptides in the treatment of various diseases and conditions. The novel peptides specifically bind to undetermined and determined targets in various organs and in lymphocytes. The subject invention also provides a method for the identification of a peptide by applying peptide library methodology <i>ex vivo</i> to perfused organs.</p>		

-1-

ISOLATION OF TISSUE SPECIFIC PEPTIDE LIGANDS
AND THEIR USE FOR TARGETING PHARMACEUTICALS TO ORGANS

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This application claims priority of U.S. Serial No. 08/810,074, filed March 4, 1997, and U.S. provisional application No. 60/039,509, filed March 4, 1997.

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BACKGROUND OF THE INVENTION

There is a need in the pharmaceutical industry for pharmaceutical agents that can be targeted to specific organs, and thus provide local drug delivery.

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Advantages of local drug delivery are the lowering of the amount of drug needed to achieve therapeutic efficacy and the minimizing of undesired side effects.

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Currently, the main approach to tissue specific targeting is either to infuse the drug through a catheter or a balloon (PTCA) to a site of the vasculature, or through linking of a drug to a protein ligand with affinity for a predetermined target.

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For example, full-size monoclonal antibodies against predetermined cell surface antigens have been generated in order to produce cell targeting ligands. However, the complexity of isolating a specific antibody and the size of such antibody are severe limitations to their use as cell

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targeting ligands.

The use of a phage peptide display library facilitates an alternative means of producing unique ligands for targeting to specific, yet unrecognized cell (undetermined) surface moieties. Phage libraries have been used to select random peptides that bind to isolated pre-determined target proteins such as antibodies, hormone receptors, and the erythropoietin receptor.

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WO 98/39469

PCT/US98/04188

-2-

Unlike the use of a known ligand, the process of phage selection from a random peptide library does not require prior knowledge of the target cell or its receptors. This approach also has the advantage that molecular recognition and ligand selection are not dependent on the immunogenicity of the candidate target protein, as required in the monoclonal antibody approach.

Pasqualini and Ruoslahti (Nature 380: 364-366, 1996) injected phage libraries intravenously into mice and subsequently rescued the phage from individual organs. Peptides capable of mediating selective localization of phage to brain and kidney blood vessels were identified and were shown to exhibit up to 13-fold higher sensitivity for these mouse organs. One of the peptides displayed by the brain-localized phage was chemically synthesized and shown to specifically inhibit the localization of the homologous phage into the brain. When coated onto glutaraldehyde fixed red blood cells, the peptide caused selective localization of intravenously injected red blood cells into the brain of the mouse.

The subject invention discloses the use of phage display peptide (epitope) libraries to identify peptides useful as ligands for targeting drugs, cells or genes to specific human tissue and various human organs, where the specific receptor is not predetermined. In addition, some experiments were carried out using predetermined targets.

The novelty of the subject approach is *inter alia* the application of the peptide library methodology to isolated perfused human tissues.

A phage library is included in the organ perfusion fluid, and after *ex vivo* organ perfusion, phages are extracted from the human tissue, amplified and the displayed peptide sequence is determined. This *ex vivo* approach is applied to

-3-

human organs such as placenta, umbilical cord artery and vein as well as blood vessels removed during surgery. This approach is further applied to diseased tissue removed during surgery and to organs such as kidney, heart and liver available following transplantation procedures.

The endothelium which lines the inner surface of blood vessels expresses multiple surface proteins and receptors for diverse types of ligands. Endothelial cells, derived from different tissues or even from veins and arteries of the same tissue, have been shown to be phenotypically and functionally distinct. The unique distinctive, characteristic surface proteins and receptors expressed by endothelial cells of the various tissues are exploited to discover novel, defined peptide ligands which are subsequently linked to drugs or radioactive isotopes for targeting to the desired tissue.

The subject invention provides a parallel approach to obtain unique, yet undetermined targets characteristic of lymphatic cells derived from different diseases, such as leukemia and autoimmune diseases. Since lymphocytes are obtained in suspension, biopanning is carried out by mixing a phage library with the lymphocyte cell suspension, followed by washing with several buffer solutions. Biopanning is repeated several times following amplification of selected phage population.

The peptide sequences of the subject invention, specific for different human organs and tissue cells are linked to various pharmaceutical agents to form drug-peptide conjugates and to radioactive isotopes for diagnostic and therapeutic purposes.

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WO 98/39469

PCT/US98/04188

-4-

Summary of the Invention

The subject invention provides novel peptides and the use of these peptides in the treatment of various diseases and conditions. The novel peptides specifically bind to
5 undetermined targets (and some determined targets) in various organs and in lymphocytes.

The subject invention provides for compositions comprising novel peptides and a pharmaceutical agent linked to the
10 peptide, wherein the pharmaceutical agent is a polypeptide and is linked to the peptide by a peptide linkage. The pharmaceutical agent may also be a toxin, an anti-cancer agent, an anti-angiogenic compound, a cardiovascular agent, an agent used in a neurological disorder, a liver disease
15 agent, a kidney disease agent or a radioisotope.

The subject invention provides for a method for the identification of a peptide comprising incubating a phage display peptide library with an isolated organ; washing the
20 isolated organ to remove unbound phages; eluting bound phage from the isolated organ; amplifying the resulting bound phage; and determining the displayed peptide sequence of the bound phage so as to identify the peptide.

25 The organ may be an artery, a vein, placenta, tumor tissue, kidney, heart, liver, or central nervous system. The organ may also be a perfused organ.

The phage display library may be a 15-mer library or a 6-mer
30 library.

The elution medium may be a compound selected from acid, urea, Octyl, trypsin or tween.

35 The subject invention also provides for a method of producing the novel peptides comprising identifying the peptide as described above; and synthesizing the peptide by joining the amino acids of the peptide in the proper order.

-5-

The subject invention additionally provides an imaging agent which comprises a peptide of the subject invention with an imageable marker. Such an imaging agent may be used for diagnostic purposes.

5

The subject invention further provides a composition comprising an effective imaging amount of an imaging agent of the invention and a physiologically acceptable carrier.

10

The subject invention also encompasses a composition comprising an effective imaging amount of an imaging agent of the invention, a pharmaceutical agent linked thereto and a physiologically acceptable carrier.

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The marker may be a radioactive isotope, an element which is opaque to X-rays or a paramagnetic ion.

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The radioactive isotope may be indium-111, technetium-99, iodine-123, iodine-125, iodine-131, krypton-81m, xenon-33 or gallium-67.

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The subject invention also provides for a method for imaging an organ comprising contacting the organ to be imaged with an imaging agent under conditions such that the imaging agent binds to the organ; imaging bound imaging agent; and thereby imaging the organ.

30

The subject invention also provides for a method of treating an organ *in vivo* comprising contacting the organ to be treated with a composition under conditions such that the composition binds to the organ; and thereby treating the organ.

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-6-

Brief Description of the FigureFigure 1: Organ distribution of selected phages

Clone KSC#3 (KSCR3#3) is a phage clone that was enriched on Kaposi Sarcoma cells after three rounds of biopanning in culture. Clone R4B*#1 (TUV-R4B*#1) is a phage clone that was enriched on umbilical vein and artery. Clone #P13 (R6P#13) is a sporadic non-enriched phage clone as a negative control. The *in vivo* binding of these three clones to tumor tissue and brain tissue was compared.

Detailed Description of the Invention

Phage display is a technique in which a peptide, antibody or protein is expressed on the surface of a bacteriophage, while the DNA encoding the displayed protein resides within the phage virion. A phage display peptide library (also termed phage peptide library or phage display library or phage library or peptide library) is constructed wherein the virions display a wide range of protein residues of specific lengths. This technology, known to one skilled in the art, is more specifically described in the following publications: Smith (1985) Science 228: 1315, Scott et al. (1990), Science 249: 386-390, Cwirla et al. (1990), P.N.A.S. 87: 6378-6382; Devlin et al. (1990), Science 249: 404-406, U.S. Patent Nos 5,427,908, 5,432,018, 5,223,409 and 5,403,484.

Biopanning is a procedure comprising many steps, one of which is selection; biopanning is carried out by incubating phages displaying protein ligand variants (a phage display library) with a target, washing away unbound phage and specifically eluting the bound phage. The eluted phage is amplified and taken through additional cycles of binding and amplification which enrich the pool of eluted specific sequences in favor of the best binding peptide bearing phages. After several rounds, individual phages are characterized, and the sequence of the peptides displayed is determined by sequencing of the corresponding DNA of the phage virion. A peptide obtained in this manner may be

-7-

called a "lead-compound".

One way of obtaining a peptide with a higher affinity relative to a lead-compound is to construct an extension phage display peptide library based on a core amino acid sequence of the lead-compound. In such an extension library, random amino acids are added to each side of the core sequence.

10 An additional way to obtain a peptide with a higher affinity relative to a lead-compound is the construction of a phagemid display mutagenesis library. In such a library, oligonucleotides are synthesized so that each amino acid of the core sequence is independently substituted by any other amino acid.

20 The subject invention provides a polypeptide which comprises a peptide of the subject invention which corresponds to a peptide displayed on a phage virion and wherein both the polypeptide and the peptide have the same biological activity.

In one embodiment, a peptide of the invention preferably has less than 50 amino acids but more than 5 amino acids.

25 In another embodiment, a peptide of the subject invention comprises 6-15 amino acids.

30 In another embodiment, to a peptide of the subject invention, 20-30 amino acids are added either to the C-terminus or the N-terminus of the peptide while the peptide maintains its biological activity.

35 In another embodiment of the subject invention, several amino acids are added either to the C-terminus or the N-terminus or to both termini of the peptide while maintaining the biological activity of the peptide. In this embodiment, 1-5 amino acids are preferably added to each terminus.

WO 98/39469

PCT/US98/04188

-8-

In an additional embodiment, a Fv fragment of a human antibody of about 100 amino acids is displayed on the N-terminus of pIII of M13 bacteriophage. In this embodiment, the peptide of the subject invention is about 100 amino acids.

Cancer tissue as used herein may be obtained from any form of cancer such as carcinoma, sarcoma, leukemia, adenoma, lymphoma, myeloma, blastoma, seminoma or melanoma.

Diseased tissue as used herein may be obtained from any diseased organ such as liver, kidney, lung, heart, ovary, colon and so forth. The organ may be diseased as a result of an autoimmune disorder. The organ may be diseased as a result of any other disease, such as cardiovascular disease or cancer.

A neurologic disorder as used herein encompasses any neurologic disorder as defined and described in "The Merck Manual", sixteenth edition (1992). For example, muscular dystrophy, myasthenia gravis, multiple sclerosis, Alzheimer's disease, neuropathy, Parkinson's disease and amyotrophic lateral sclerosis (Lou Gehrig's disease) are neurologic disorders.

A vein as used herein may originate from any tissue. An example of a vein is safenal vein or femoral vein.

An artery as used herein may originate from any tissue, e.g. radial artery, coronary artery, mammary artery and so forth.

A peptide of the subject invention may be administered to a patient, alone, radiolabeled, linked to a pharmaceutical agent (drug), or in the form of a peptidomimetic.

The mode of administration of a peptide of the subject invention is intravenous, intramuscular, subcutaneous, topical, intratracheal, intrathecal, intraperitoneal,

-9-

rectal, vaginal or intrapleural.

The pharmaceutical agent may *inter alia* be a radioactive label (radio-isotope).

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If the peptide or the peptide-drug combination is administered orally, it is administered in the form of a tablet, a pill or a capsule.

10 The compositions comprising the peptides produced in accordance with the invention may comprise conventional pharmaceutically acceptable diluents or carriers. Tablets, pills and capsules may include conventional excipients such as lactose, starch and magnesium stearate. Suppositories
15 may include excipients such as waxes and glycerol. Injectable solutions comprise sterile pyrogen-free media such as saline and may include buffering agents, stabilizing agents or preservatives. Conventional enteric coatings may also be used.

20

Compositions for topical administration may be in the form of creams, ointments, lotions, solutions or gels.

The mode of administration of the peptide or drug-peptide
25 linkage is a solid dosage form, a liquid dosage form, or a sustained-release formulation.

The subject invention provides peptides comprising amino acids having the following sequences:

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Gly Arg Gln His Phe Phe Leu Ala Glu Gly Arg Ser Phe Tyr Phe; 1

Ser His Val Pro Pro Ile Phe Asn Asp Val Tyr Trp Ile Ala Phe; 2

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Val Pro Pro Ile Phe Asn Asp Val Tyr Trp Ile Ala Phe; 3

His Thr Phe Phe Leu Pro Gly Cys Ala Gly His Cys Ile Asp Ala; 4

WO 98/39469

PCT/US98/04188

-10-

Pro Ser Thr Thr Arg Asn Arg Thr Asp Ile Asn Lys Pro Thr Gln; 5

Phe Tyr Ser His Ser Ala Asp Gly Ala Arg Pro Phe Pro Leu Tyr; 6

5 Phe Tyr Ser His Ser Ala Asp Gly Ala Glu Ser Ser Pro Arg Met; 7

Phe Tyr Ser His Ser Ala Asp Gly Ala Pro Arg Arg Asp Leu Leu; 8

Phe Tyr Ser His Ser Ala Asp Gly Ala; 9

10 Phe Tyr Ser His Ser Ala Asp Gly Ala XXX XXX XXX Pro XXX XXX, 10
wherein XXX is any amino acid;

Phe Tyr Ser His Ser Ala Asp Gly Ala XXX XXX XXX XXX Leu XXX, 11
15 wherein XXX is any amino acid;

Gly Cys Gly Gly Phe His Pro Gly Ser Asn Cys Leu Val Val Ser; 12

Arg Asn Val Pro Pro Ile Phe Asn Asp Val Tyr Trp Ile Ala Phe; 13

20 Phe Leu Pro Asn Gly Phe; 14

Thr His Asp Thr His Leu; 15

25 Thr His Glu Thr Gln Arg; 16

Asp Ile Ala Lys Arg Tyr; 17

Asp Val Ser Pro Val Trp Ala Ala Phe Ala Ser Gly Ala Ser Phe; 18

30 Ser Asp Cys Leu His Ser Val Arg Gly Phe Asn Cys Leu Lys Arg; 19

Ser Leu Cys Phe Tyr Leu Phe Val Met Ser Ala Pro Asp Ala Pro; 20

35 Gly Leu Gly Gly Leu Ser Phe Gly His Ser Asp Asn Pro Pro Ser; 21

Gly Pro Gly Trp Val Gly Trp Phe Val Ser Leu Phe Tyr Ala Ser; 22

WO 98/39469

PCT/US98/04188

-11-

Trp Ser Leu Gly Ser Ser Trp Val Tyr Lys Phe Phe Tyr Ser Ser;
 Ser Leu Trp Gly Ala Ser Ser Cys Gly Val Ala Phe Phe Glu Ser;
 Gly Pos Pos Pos/Ar Ar Ar Leu Ala Glu Gly Arg Ser Ar Ar Ar
 5 wherein Pos is a positively charged amino acid and Ar is an
 aromatic amino acid.
 Arg Ser Lys Tyr Arg Pro Asn Met Thr Asn
 10 Leu Asn Pro Lys Val Lys His Met
 Leu Arg Gly Gly Asn Ala Met
 Val Ser Asp Arg Arg Gln Asn Val
 15 Ser Lys Ser Pro
 Gly Thr Leu Asn Gln Cys Gly Arg Ile Asn
 20 Cys Ala Val Glu Ala Ala Gly Pro Val Arg Val Leu
 Ser Gly Ser Leu Gly Arg Ser Leu Glu
 Thr Gly Asp Glu
 25 Phe Lys Ala Ser Arg His Ser
 Ile His Met Arg Ala
 30 Lys Asn Ala Asn
 Met Arg Ala Pro Val Ile
 35 Gly Ile Lys Gly Leu Asp Glu
 Cys Lys Trp Glu Lys Arg

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WO 98/39469

PCT/US98/04188

-12-

Ala Arg Leu Ser Pro Thr Met Val His Pro Asn Gly Ala Gln Pro 40

Ala Leu Gly Gly Phe Arg Pro Phe Trp Ser Tyr Gly Gly Leu Ser 41

5 Met Gly Ala Asp Asp Ala Pro His Tyr Trp His Pro Val Trp Thr 42

Cys Thr Arg Leu Gly Ala Ala Ala Gly Arg Cys Asp Val Gly Leu 43 { ?

Arg Leu Phe Met Leu Gly

10 Pro Ile Trp His Gly Asp Ser Gly Val Tyr Ser Ser Phe Phe Pro 44

Arg Asn Thr Leu Pro His Phe Ser Phe Gly Pro Arg Leu Tyr Arg 45

15 Gly Leu Ser Asp Gly pro Tyr Tyr Ser Phe Ser Leu Phe Arg Phe 46

Gly Gly Ala Ala Gly Gly Tyr Leu Arg Val Phe Ala Gly Val Arg 47

Gly Tyr Asp Cys Trp Asp Cys Pro Phe Ser Phe Arg Gly Ser Val 48

20 Ser Asn Leu Asn Arg Phe Val Phe Ala Phe Trp Asp Gly Pro Ala 49

Leu His Gly Phe Ala Ser His Lys Asp Gly Pro Leu Ile Pro Ala 50

25 Leu Val Phe Val Lys Asn His Pro Leu Val Pro Phe Gly Ser Pro 51

Ser Lys Arg Ala Asn Gly Phe Arg Gly Val Ser 52

S L R A N G F R G V S 53

Ile Lys His Tyr Gly Arg Lys Arg Asn 54

30 I K H Y G R K R N

Val Lys Lys Phe Lys Gly Gly Gln Arg Val

V K K F K G G Q R V

In each of the sequences in this application the amino acid at the extreme left represents an amino acid on the amino terminal side of the peptide and the amino acid at the extreme right represents an amino acid on the carboxy terminal side of the peptide.

-13-

Peptides which comprise the above sequences are also encompassed by the subject invention.

5 The invention further provides a composition comprising a peptide of the subject invention and a pharmaceutical agent linked thereto. The composition optionally also comprises a pharmaceutically acceptable carrier.

10 In a preferred embodiment, the pharmaceutical agent is a polypeptide and is linked to the peptide by a peptide linkage.

15 In another embodiment, the pharmaceutical agent is a toxin, an anti-cancer agent, an anti-angiogenic compound, a cardiovascular agent, an agent used in a neurological disorder, a liver disease agent or a kidney disease agent or a radio isotope.

20 In a presently preferred embodiment, the pharmaceutical agent is a recombinant protein.

25 The subject invention further provides a composition comprising a peptide of the subject invention and a pharmaceutically acceptable carrier.

The subject invention also provides a chimeric polypeptide comprising a first peptide and a second peptide wherein the first peptide is a peptide of the subject invention.

30 In a preferred embodiment, the second peptide is a toxin, an anti-cancer agent, an anti-angiogenic compound, a cardiovascular agent, an agent used in a neurological disorder, a liver disease agent or a kidney disease agent.

35 In another preferred embodiment, the second peptide is a recombinant protein.

The subject invention also provides a method for the

WO 98/39469

PCT/US98/04188

-14-

identification of a peptide which comprises incubating a phage display peptide library with cells of an isolated organ, washing the isolated organ to remove unbound phages, eluting bound phage from the isolated organ, amplifying the
5 resulting bound phage and determining the displayed peptide sequence of the bound phage so as to identify the peptide.

The subject invention also encompasses a method of synthesizing a peptide of the subject invention which
10 comprises joining the amino acids of the peptide in the proper order.

The subject invention further provides a method of producing a peptide which comprises identifying the peptide by the
15 above described method and synthesizing the peptide by joining the amino acids of the peptide in the proper order.

In a preferred embodiment, the isolated organ is a perfused organ.
20

In another embodiment, the isolated organ is an artery, a vein, placenta, tumor tissue, kidney, heart, liver or central nervous system.

25 In yet another embodiment, the artery is umbilical cord artery, a radial artery, a coronary artery, a mammary artery or a damaged artery.

In an especially preferred embodiment, the damaged artery is
30 a damaged coronary artery.

In a preferred embodiment, the vein is umbilical cord vein, safenal vein or femoral vein.

35 It is envisaged that the phage display peptide library is a 15-mer library, a 6-mer library or a synthetic human antibody library.

-15-

Preferred elution medium is a compound selected from acid, urea, Octyl, trypsin or tween.

5 The subject invention additionally provides an imaging agent which comprises a peptide of the subject invention with an imageable marker. Such an imaging agent may be used for diagnostic purposes.

10 The subject invention further provides a composition comprising an effective imaging amount of an imaging agent of the invention and a physiologically acceptable carrier.

15 The subject invention also encompasses a composition comprising an effective imaging amount of an imaging agent of the invention, a pharmaceutical agent linked thereto and a physiologically acceptable carrier.

20 In a preferred embodiment, the marker is a radioactive isotope, an element which is opaque to X-rays or a paramagnetic ion.

25 In a presently preferred embodiment, the radioactive isotope is indium-111, technetium-99, iodine-123, iodine-125, iodine-131, krypton-81m, xenon-33 or gallium-67.

30 The subject invention also provides a method for imaging an organ which comprises contacting the organ to be imaged with an imaging agent of the invention under conditions such that the imaging agent binds to the organ, imaging the bound imaging agent and thereby imaging the organ.

In a preferred embodiment, the organ is an artery, a vein, placenta, tumor tissue, kidney, heart or liver.

35 In another embodiment, the artery is umbilical cord artery, a radial artery, a coronary artery, a mammary artery or a damaged artery.

WO 98/39469

PCT/US98/04188

-16-

In another embodiment, the damaged artery is a damaged coronary artery.

5 In another embodiment, the vein is umbilical cord vein, safenal vein or femoral vein.

10 In another embodiment, the pharmaceutical agent is a polypeptide and is linked to the imaging agent by a peptide linkage.

15 In a preferred embodiment, the pharmaceutical agent is a toxin, an anti-cancer agent, an anti-angiogenic compound, a cardiovascular agent, an agent used in a neurological disorder, a liver disease agent or a kidney disease agent.

The subject invention further provides a composition wherein the pharmaceutical agent is a recombinant protein.

20 The subject invention further provides a method of treating an organ *in vivo* which comprises contacting the organ to be treated with a composition of the invention under conditions such that the composition binds to the organ and thereby treating the organ.

25 In a preferred embodiment, the organ is an artery, a vein, placenta, tumor tissue, kidney, heart, liver, or central nervous system.

30 In another embodiment, the artery is umbilical cord artery, a radial artery, a coronary artery or a mammary artery or a damaged artery.

In another embodiment, the damaged artery is a damaged coronary artery.

35 In another embodiment, the vein is umbilical cord vein, safenal vein or femoral vein.

WO 98/39469

PCT/US98/04188

-17-

The novel peptides of the subject invention or their corresponding peptidomimetics are also used in the manufacture of compositions to treat various diseases and conditions.

5

The subject invention also provides a method for the identification of peptides or antibodies by biopanning which comprises incubating a phage display library with lymphocytes derived from blood, washing to remove unbound phages, eluting the bound phages from the lymphocytes, 10 amplifying the resulting bound phage and determining the displayed peptide sequence of the bound phage so as to identify the peptide.

WO 98/39469

PCT/US98/04188

-18-

Examples

The Examples which follow are set forth to aid in understanding the invention but are not intended to, and should not be construed to, limit its scope in any way.

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EXAMPLE 1: Isolation and Selection of Tissue-Specific Epitopes which Specifically Bind to Undetermined Targets in Umbilical Cord Vein and Artery

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1. MATERIALS AND METHODS

1.1. Preparation of perfused umbilical cord vein and artery
Freshly obtained full term umbilical cord was cut into fragments of 4-5 cm in length, the blood was removed and the umbilical cord was cannulated and connected either through the vein (V) or the artery (A) to a small circulating pump for perfusion at a rate of 1ml/min (Pharmacia peristaltic pump). Perfusion temperature was either 4°C or 23°C and the perfusion buffer composition is indicated below.

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1.2. Peptide display phage library source

Two phage display peptide libraries (6-mer and 15-mer) were kindly provided by G. Smith (Virology 167: 156-165, 1988). The libraries were amplified to form phage library working stock.

25

The libraries were originally constructed by splicing the 2.8 kilo-base pair tetracycline resistance determinant of Tn10 into the minus-strand origin of replication of the wild type fd phage. The resulting defect in minus strand synthesis reduced the intracellular replicative form (RF) copy number to 1. As a result thereof, the filamentous phage including fd-tet do not kill their host. The infected cell, which becomes resistant to tetracycline, continues to grow and secret tetracycline resistant progeny particles (about 10 tetracycline transducing units (TTU) per bacteria). The infectivity of the 6-mer and 15-mer libraries are 2.5% and 39%, respectively. The calculated theoretical

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WO 98/39469

PCT/US98/04188

-19-

number of primary clones for the 6 and 15-mers libraries are 4×10^7 and 1×10^8 , respectively. However, the number of primary clones obtained from each library was about 2×10^8 . The phage titer is determined by infecting starved K91/kan bacterial culture and selecting tetracycline resistant clones on kan/tetracycline agar plates. The biopanning yield is calculated as the % of the output phage from the total input.

10 **1.3. Bacterial strain: K91/Kan**

The preparation of starved bacterial culture for phage infection was essentially as described by G. Smith (1993), Methods of Enzymology 217:228.

15 **1.4. Phage selection and amplification**

Phages that expressed epitopes of specific interest were selected from the libraries by biopanning in an essentially 4-step procedure:

- 20 a. Binding of phage to the perfused vessel cells
 b. Removal of non-bound phage by extensive washing
 c. Elution of bound phages
 d. Infection and propagation of eluted phage in *E. coli*.

25 This biopanning procedure was generally repeated 4-6 times. Selected clones were individually propagated, and the single stranded DNA from the secreted phage was purified. Properties of the selected clones were examined as follows:

30 (i) Sequencing selected phage:

The DNA sequence of the insert was determined by the dideoxy DNA sequencing method (Sanger et al. (1977), P.N.A.S. 74: 5463-5467) using Sequenase Version 2.0 (DNA Sequencing Kit, Amersham) and a primer of 18 nucleotides (5': TGAATTTTCTGTATGAGG).

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(ii) Biopanning and tissue distribution in vivo (mouse or rat)

WO 98/39469

PCT/US98/04188

-20-

- (iii) Phage binding comparison *ex vivo*, in culture, and *in vivo*

The following procedures are then carried out (Example 4):

- 5
- (iv) Peptide synthesis
- (v) Mutant peptide synthesis
- (vi) Peptide cross linking (chemically and by genetic engineering)
- 10 (vii) Immunolocalization (using labeled antibody)
- (viii) Linking of peptide to drug
- (ix) Radiolabeling of peptide
- (x) Radiotherapy and other therapeutic treatments.
- (xi) Organic synthesis of peptidomimetic.
- 15

1.5 Basic Biopanning protocols

Several biopanning protocols were developed and used:

- 1.5.1. Protocol T1 (Acid/Urea/Octyl elution)
- 20 1.5.2. Protocol R1 (Trypsin-EDTA/Acid-Tween elution)
- 1.5.3. Protocol N1 (Acid-Tween/Tissue elution)
- 1.5.4. Protocol R2 (Trypsin-EDTA/Acid-Tween/Tissue elution)
- 1.5.5. Protocol EC-1 (Acid/Tissue/Urea elution)

- 25 1.5.1. Protocol T1 (Acid/Urea/Octyl elution)

1. Prewashing of the umbilical cord was carried out with 20 ml cold PBS¹ followed by 20ml cold DMEM-5% BSA.

2. Selection was carried out at 2×10^{10} TTU² in 3ml, DMEM³-1%

¹ PBS is 136 mM NaCl, 2.7 mM KCl, 100 mM Na₂HPO₄, 2H₂O, 1.4 mM KH₂PO₄

² TTU is phage infection units, conferring tetracycline resistance in the *E. coli* host.

³ DMEM is Dulbecco's Modified Eagle Medium with D-glucose (450 mg/l), Bet HaEmek

WO 98/39469

PCT/US98/04188

-21-

BSA⁴-Protease inhibitors mixture ⁵(Pi). (50 μ l of the selection solution is used for titration of the input phage).

5 3. Wash 1+ Wash 2 (W1,W2) was carried out in 2 x 25ml DMEM-5% BSA-Pi. When indicated, 0.2% Tween-20 was included.

10 4. Wash 3 (W3) was carried out in 3 ml W1+2 containing TBS⁶-Octyl⁷(0.05%).

Steps 2-4 were carried out at 4°C or at 23°C as indicated below.

15 5. Elutions: eluates were collected into 6ml TBS-1% BSA and adjusted to pH 7.4 with Trisma Base.

5a. Acid elution was carried out in 3 ml 0.2M glycine-HCl, pH 2.2.

20 5b. Urea elution was carried out in 3 ml 6M urea, pH3 (in 0.2M Glycine-HCl, pH3.0).

5c. Octyl elution was carried out in 3ml TBS containing 0.2% Octyl.

25

6. Concentration/dialysis of the eluates was carried out on a 30K millipore dialysis membrane.

30 7. Amplification: for the first biopanning round, the entire eluate was amplified. For other rounds of biopanning only 20% of the eluate was amplified. The amplification was

⁴ BSA is bovine serum albumin fraction V (Sigma)

⁵ Pi is Phenyl Methyl Sulfonyl Fluoride (PMSF) 1mM, Aprotinin (20 μ g/ml), Leupeptin (1 μ g/ml)

⁶ TBS is 50 mM Tris-HCl, pH 7.5 and 150 mM NaCl

⁷ Octyl is octyl- β -D-glucopyranoside (Sigma)

-22-

carried out in liquid medium by mixing the eluate with equal volume of 10^{10} starved bacteria. After 10 minutes, 100ml NZY⁸ solution containing 0.2 μ g/ml tetracycline was added and bacterial suspension was mixed vigorously at 37°C for 30 minutes. Diluted samples were plated immediately on agar/kanamycin/tetracycline plates for titration of the output phage. To the rest of the bacterial cell suspension 100 μ l of 20mg/ml tetracycline were added and incubation/amplification continued over-night.

10

1.5.2. Protocol R1 (Trypsin-EDTA/Acid-Tween elution)

15

1. Prewashing of umbilical cord vein and artery was carried out with 30ml DMEM-heparin (5u/ml) (Laboratoire Choay)- 10^8 M13 phage followed by 30ml, DMEM-5% human serum (Sigma).

20

2. Selection was carried out with 30ml, DMEM-5% human serum containing phage, 2×10^{10} TTU.

25

3. Wash was carried out with 40ml DMEM 5% human serum in the presence of Pi.

30

4. Elutions were carried out with:

- a. Trypsin-EDTA (2 ml, 0.25% and 0.05% respectively).
- b. Acid-Tween (5ml, 0.2M glycine-HCl (pH2.2)-0.5% Tween).

35

5. Titration and amplification:

40

The acidic fraction was neutralized with 2M Tris base (about 270 μ l). To the combined 7ml eluate, 200 μ l, 10^{10} bacteria and 10ml NZY were added. The solution was mixed gently for 10 minutes at 37°C. 100ml prewarmed NZY and 0.2 μ g/ml tetracycline was added and mixed vigorously for 30 minutes. 100 μ l 20mg/ml tetracycline was added and steps 1-4 were continued on ice and step 5 at room temperature.

45

1.5.3. Protocol N1 (Acid-Tween/Tissue elution)

⁸

NZY is a medium consisting of 10 g NZ amine A, 5 g yeast extract and 5 g NaCl.

WO 98/39469

PCT/US98/04188

-23-

1. Prewashing was carried out with 30ml DMEM-heparin (5u/ml) containing 10^8 M13 phage followed by 30ml DMEM-5% human serum.

5 2. Selection was carried out with 30ml DMEM-5% human serum, containing 4×10^{10} TTU.

3. Wash was carried out with 40ml DMEM-5% human serum and 2% human haemoglobin (Sigma)-Pi.

10

4. Elution 1 was carried out in 3ml acid (0.2M glycine-HCl (pH2.2), 1% BSA, 0.5% Tween-20). The blood vessel was further washed with 2ml DMEM-5% human serum and combined with elution 1. 200 μ l 10^{10} bacteria and 10ml NZY were added, mixed gently and left at room temperature for 10 minutes.

15

Elution 2, tissue elution (i.e. bacteria elution), was carried out by clamping one side of the vein and adding 0.5ml NAP⁹ buffer containing 2.5×10^8 starved bacteria. After clamping the other end of the vein, the blood vessel was immersed into DMEM- 1% BSA solution and shaken at 37°C for 45 minutes. The bacteria were removed to a 50ml tube, the blood vessel was washed twice with 1-2ml NAP buffer, and the two washes were combined. 10ml NZY was added, mixed gently and left at room temperature for 10 minutes.

20

25

Steps 1-4 were carried out on ice and step 5 at room temperature (using a lamp from above).

30

5. Titration and amplification:

100ml prewarmed NZY containing 0.2 μ g/ml tetracycline was added and mixed vigorously for 30 minutes. Samples (50 μ l each) were removed for titration on plates (output), and for over-night amplification, 100 μ l of 20mg/ml tetracycline was added.

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⁹ NAP consists of 80mM NaCl and 50 mM $\text{NH}_4\text{H}_2\text{PO}_4$, pH 7.0

WO 98/39469

PCT/US98/04188

-24-

1.5.4. Protocol R2 (Trypsin-EDTA/Acid-Tween/Tissue elution)
Protocol R2 is essentially identical to protocol R1 apart from the addition of tissue elution with bacteria following the trypsin and acid-tween elutions.

5

1.5.5. Protocol EC-1

Primary human endothelial cells seeded in 35cm tissue culture bottles (3rd passage of full term umbilical cord) were used.

10

1. Re-equilibration to 37°C and CO₂ for 30 minutes.

15

2. Prewashing was carried out with 5ml serum-free medium followed by 10 ml blocking solution (DMEM-BSA(1%)-Pi) for 90 minutes at 37°C.

20

3. Selection (incubation) for 45 minutes at 37°C with 3ml DMEM-BSA(1%)-Chloroquine (100µM) mixture containing 2x10¹⁰ phages of the 15-mer initial library and a selected phage clone at a ratio of approximately 100:1, respectively.

4. Washing was carried out 5 times with 5ml blocking solution for 5 minutes each at room temperature.

25

5. Elutions:

30

5a. Elution 1 was carried out with 2ml acid-glycine pH 2.2 containing 0.2% Tween for 10 minutes at room temperature. 170µl of 2M Trizma base was added to the bottle for neutralization, and the acid elution obtained was transferred to another tube. The bottle was washed with 2ml DMEM-BSA(1%). 0.2ml of the eluate was mixed with NAP buffer containing 1x10¹⁰ starved bacteria, and after 10 minutes absorption at room temperature, 2ml NZY-0.2% tetracycline was added and the suspension was shaken at 37°C. Aliquots were plated on agar-kanamycin-tetracycline plates for phage titration.

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5b. Elution 2 was carried out with 2ml NaPO₄ buffer

WO 98/39469

PCT/US98/04188

-25-

containing 5×10^{10} starved bacteria. Incubation was carried out at room temperature for 45 minutes on a rocker. Cell eluate was transferred to another tube. The bottle was washed with 2ml medium. 0.4ml suspension was mixed with 2ml
5 NZY 0.2%-tetracycline and incubated and titrated as described for elution 1(6a).

5c. Elution 3 was carried out with 2ml urea (6M, pH 3). Incubation at room temperature for 10 minutes while rocking.
10 170 μ l of 2M Trizma base was added directly to the bottle and urea eluate was transferred to another tube containing 36ml medium. The bottle was washed with 2ml medium. 0.2ml was mixed with 0.2ml NAP buffer containing 1×10^{10} starved bacteria and then continued as described for elution 1(6a).

15

Testing the working stock of 15-mer library:

The working stock of the 15-mer library was the product of several cycles of amplifications. Several clones of the working stock (i.e. input of round 1) were isolated,
20 amplified and their single stranded DNA was purified and sequenced. None of the phage clones was identical to specifically selected clones described below.

In addition, approximately 500 colonies derived from the
25 same library were examined by colony hybridization to the 32 P-labeled antisense oligonucleotide probe of clones TUV-R4B-#1 and TUV-R4B-#3 disclosed below. None of the colonies hybridized to these probes.

-26-

2. RESULTS:

2.1. Specificity of phage library to umbilical cord

Table 1: Comparison of phage binding to umbilical cord vein and non-specific phage adsorption to peristaltic pump plastic tubes following biopanning with 15-mer library (yield after acid and urea elution, protocol T1, room temperature (23°C)).

	Plastic tubes ¹ (TTU)	yield %	<u>Vein</u> ² (TTU)	yield %
input	2×10^9		3×10^{10}	
W1 + Tween	1×10^8	6	1×10^9	3.8
W2 + Tween	2×10^6	0.1	9×10^6	0.03
WO	2×10^5	0.001	1×10^5	0.0004
acid elution	$<< 10^3$	$< .00001$	7×10^6	0.02
urea elution	$<< 10^3$	$< .00001$	9×10^6	0.0260

¹ Selection was carried out through the circulating pump without the umbilical vein.

² Selection was carried out through the umbilical vein and the circulating pump.

Thus, the results shown in Table 1 indicate that the phage yield values of the acid and the urea elutions from the umbilical vein were 2 to 3 orders of magnitude higher than the background values obtained from background binding to the plastic tubes, thus indicating a specific binding of the phage to the umbilical vein.

WO 98/39469

PCT/US98/04188

-27-

2.2 Effect of temperature and detergent on phage binding
Table 2: Effect of temperature and Tween-20 on the binding
 of the 6-mer and 15-mer phage libraries to umbilical artery
 and vein (acid/urea/Octyl elution, protocol T1).

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	<u>Artery</u> (4°C), 6-mer- (TTU)	<u>Vein 1</u> (4°C), 15-mer- (TTU)	<u>Vein 2</u> (23°C), 15-mer (TTU)
Input	2.6×10^{10}	2.5×10^{10}	1.8×10^{10}
W1	1.7×10^{10}	2.5×10^{10}	
W2	1.4×10^9	2.8×10^9	
W1+Tween			1.3×10^{10}
W2+Tween			9×10^6
WO	2.8×10^8	6.5×10^8	1.3×10^5
Acid Elution	3.1×10^6	1.5×10^6	6.6×10^6
Urea Elution	3.0×10^6	3×10^6	9.0×10^6
Octyl Elution	3×10^8	2.4×10^8	1.1×10^6

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The results indicate that by the addition of Tween (0.2%) in
 the washing solution and by increasing the temperature of
 the biopanning procedure to 23°C, there is a dramatic
 decrease in phage titers of the subsequent washing and
 elution steps (i.e. W2 + Tween, WO and Octyl elution) both
 with artery and vein. Therefore, the conditions of vein 2
 (i.e. biopanning at 23°C and washing with a buffer containing
 0.2% Tween followed by washing with a buffer containing
 0.05% Octyl) were selected and used in the following
 protocol T1 based experiments.

-28-

2.3 Yield of Biopanning with Umbilical Vein and Artery

5 Table 3: Biopanning of 15-mer library with human umbilical vein (Vein 1 and Vein 2) and 6-mer library with human umbilical artery (Artery 1) - yield after urea elution, protocol T1 (experiment B).

Round #	Artery 1 (4°C) (TTU)	Vein 1 (4°C) (TTU)	Vein 2 (23°C) (TTU)
^a R1B	1.7×10^{-2}	2×10^{-2}	2.3×10^{-2}
^b R2B	0.9×10^{-2}	0.8×10^{-2}	2×10^{-2}
10 ^c R3B	1.2×10^{-1}	1×10^{-1}	1.3×10^{-1}
^d R4B	1.5×10^{-1}	1.3×10^{-1}	1.28×10^{-1}
^e R4B*	-	-	3×10^0

15 The protocol used was essentially as protocol T1 with indicated modifications below. Input phage in rounds R1B-R4B was 2×10^{10} - 10^{11} and in R4B* was 1×10^9 .

- 20 a. Selection was carried out for 15 minutes. Wash was carried out in buffer without Tween, followed by buffer containing Octyl.
- 25 b. Selection was carried out for 8 minutes. Vein 2 was washed with buffer containing Tween, followed by buffer containing Octyl. Artery 1 and Vein 1 were washed with buffer without Tween followed by buffer containing Octyl. Elutions were carried out for 8 minutes.
- 30 c. Selection was carried out for 3 minutes. Washing was carried out with buffer containing Tween followed by buffer containing Octyl for all blood vessels. Elution was carried out for 8 minutes.
- d. and e. Selection and washing conditions were identical to

WO 98/39469

PCT/US98/04188

-29-

c. Elution was carried out for 3 minutes.

Twenty-eight clones were sequenced after R4B*V2, i.e. the urea eluted fraction from umbilical vein 2 after round 4:

5

17/28 (60%) of the clones were identical: this clone was designated TUV-R4B*--#1:

Amino Acid Sequence displayed by clone TUV-R4B*--#1:

10 Gly Arg Gln His Phe Phe Leu Ala Glu Gly Arg Ser Phe Tyr Phe
 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

15 Six amino acids are aromatic (bolded) forming two hydrophobic clusters (amino acids 4,5,6,...13,14,15), accompanied by a linker peptide (underlined) region containing the charged sequence Glu Gly Arg (9,10,11) followed by Ser Phe. This sequence is similar to the NGRSF (Asn Gly Arg Ser Phe) sequence motif obtained by selection of phage bound to the $\alpha 5\beta 1$ integrin (E. Koivunen et al., 20 1994, Methods in Enzymology 245: 346-367). In addition, the amino end of this peptide is positively charged having the sequence Arg, Gln, His (position 2, 3, 4, double underlining).

25 3/28 (11%) identical clones were designated TUV-R4B*--#3:

Amino Acid Sequence displayed by clone TUV-R4B*--#3:

30 Ser His Val Pro Pro Ile Phe Asn Asp Val Tyr Trp Ile Ala Phe
 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

The underlined sequence also appears in clone TUV-R5D-#2 (see below).

35 2/28 (7%) were identical clones and were designated TUV-R4B*--#11.

WO 98/39469

PCT/US98/04188

-30-

Amino Acid Sequence displayed by clone TUV-R4B*--#11:

<u>His</u>	<u>Thr</u>	<u>Phe</u>	<u>Phe</u>	<u>Leu</u>	<u>Pro</u>	<u>Gly</u>	<u>Cys</u>	<u>Ala</u>	<u>Gly</u>	<u>His</u>	<u>Cys</u>	<u>Ile</u>	<u>Asp</u>	<u>Ala</u>
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15

5 2/28 (7%) were identical clones and were designated TUV-R4B*--#23.

Amino Acid Sequence displayed by clone TUV-R4B*--#23:

<u>Pro</u>	<u>Ser</u>	<u>Thr</u>	<u>Thr</u>	<u>Arg</u>	<u>Asn</u>	<u>Arg</u>	<u>Thr</u>	<u>Asp</u>	<u>Ile</u>	<u>Asn</u>	<u>Lys</u>	<u>Pro</u>	<u>Thr</u>	<u>Gln</u>
10 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15

4/28 were unique clones.

15 Furthermore, ten clones were sequenced after R4BV2, i.e. another urea eluted fraction from umbilical vein 2 after round 4:

3/10 (30%) clones were identical to clone TUV-R4B*--#1 and 7/10 were unique clones.

20

In addition, seven clones were sequenced after R4BA1, i.e. the urea eluted fraction from umbilical artery after round 4: all seven clones were unique clones.

-31-

Table 4: Biopanning of 15-mer library with umbilical vein - yield of acid elution fraction using Protocol T1 (Experiment B)

Round#	Input (TTU)	Output (TTU)	Yield(%)
R3Ba ¹	3×10^{10}	7×10^6	2×10^{-2}
R4Ba	7×10^9	5×10^5	6×10^{-3}
R5Ba	4×10^{10}	2×10^6	6×10^{-3}
R6Ba	3×10^{10}	3×10^6	9×10^{-3}

¹ The amplified acid elution of R2B-V2 (Table 3) was used as the input of round 3. In the subsequent rounds (R4Ba-R6Ba) the amplified acid eluted phage was used for biopanning.

Ten clones were sequenced after R6Ba, i.e. the acid eluted fraction from umbilical vein 2 after round 6:

4/10 clones were identical and displayed strong homology to clone TUV-R4B*-#3. This clone was designated TUV-R6Ba-#7.

Amino acid sequence displayed by TUV-R6Ba-#7:

Arg Asn Val Pro Pro Ile Phe Asn Asp Val Tyr Trp Ile Ala Phe
 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

The bolded amino acid sequence appears in clone TUV-R4B*-3# as well. Moreover, this clone is identical to UV-R5D-#2 (below). Thus, TUV-R6Ba = TUV-R5D-#2.

In addition, 1/10 clone was identical to TUV-R4B*-#1.

WO 98/39469

PCT/US98/04188

-32-

Table 5: Biopanning of 15-mer library with umbilical vein -
yield of Octyl elution fraction using Protocol T1
(Experiment B)

Round #	Input (TTU)	Output (TTU)	Yield (%)
R3BO ²	3×10^{10}	1×10^6	3×10^{-3}
R4BO	3×10^{10}	5×10^6	2×10^{-3}
R5BO	2×10^{11}	7×10^5	4×10^{-4}
R6BO	1×10^{10}	4×10^5	4×10^{-3}

² The amplified Octyl eluate of R2B-V2 (Table 3) was used as the input of round 3. In the subsequent rounds (R4BO-R6BO) the amplified Octyl eluted phage was used for biopanning.

Seven clones were sequenced after R4BO, i.e. the Octyl eluted fraction from umbilical vein 2 after round 4:

6/7 (85%) clones were identical to clone TUV-R4B*-#1.

-33-

Table 6: Biopanning of 15-mer library with umbilical vein - yield after acid-tween/trypsin elution, protocol R1 (experiment C):

5 I. Yields of Rounds 1-5 with umbilical vein

	Input (TTU)	Output (TTU)	Yield(%)
R1C-Vein	5×10^8	1.5×10^6	0.03
R2C-Vein	3.3×10^{10}	2.8×10^6	0.0085
R3C-Vein	4.7×10^{10}	6.7×10^5	0.0014
10 R4C-Vein	2.4×10^{10}	8.4×10^5	0.0035
R5C-Vein	1.3×10^{10}	4.9×10^6	0.037

II. Yields of Round 6 with umbilical vein and artery.

	Input (TTU)	Output (TTU)	Yield (%)
15 R6C-Vein	1.5×10^{11}	6.4×10^6	0.004
R6C-Artery	1.5×10^{11}	1.7×10^6	0.001

20 Seven clones were sequenced after R4C, i.e. the Acid-Tween/Trypsin eluted fraction from umbilical vein after round 4:

6/7 (85%) clones were identical to clone TUV-R4B*--#1.

25 Furthermore, seven clones were sequenced after R5C, i.e. the Acid-Tween/Trypsin eluted fraction from umbilical vein after round 5:

7/7 clones were identical to clone TUV-R4B*--#1.

30

The binding of clone TUV-R4B*--#1 to the umbilical vein (R6C-V) and to the artery (R6C-A) was nearly identical, indicating the inability of clone TUV-R4B*--#1 to discriminate between the two types of vessels.

WO 98/39469

PCT/US98/04188

-34-

Table 7: Biopanning of umbilical vein with a mixture of the 6-mer and the 15-mer libraries (10^{10} TTU each)- yield of tissue elution with protocol N1 (Experiment D)

Round #	Input (TTU)	Output (TTU)	Yield (%)
R1D	2×10^{10}	2×10^7	10^{-1}
R2D	5×10^{10}	4×10^7	8×10^{-2}
R3D	3×10^{11}	2×10^6	10^{-3}
R4D	2×10^{10}	8×10^6	4×10^{-2}
R5D	3×10^{10}	7×10^6	3×10^{-2}

Six clones were sequenced after R4D, i.e. the tissue eluted fraction from umbilical vein after round 4:

3/6 clones (clones #1, #2, and #4) were identical at nucleotide positions 1-27 (amino acids 1-9). Clones #1 and #2 also have an identical amino acid at position 13 (proline) and clones #1 and #4 have an identical amino acid at position 14 (leucine).

These clones were designated TUV-R4D-#1, TUV-R4D-#2, and TUV-R4D-#4.

Amino Acid Sequence displayed by clone TUV-R4D-#1

Phe Tyr Ser His Ser Ala Asp Gly Ala Arg Pro Phe Pro Leu Tyr
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Amino Acid Sequence displayed by clone TUV-R4D-#2:

Phe Tyr Ser His Ser Ala Asp Gly Ala Glu Ser Ser Pro Arg Met
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Amino Acid Sequence displayed by clone TUV-R4D-#4:

Phe Tyr Ser His Ser Ala Asp Gly Ala Pro Arg Arg Asp Leu Leu
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

WO 98/39469

PCT/US98/04188

-35-

The amino acids bolded in clones TUV-R4D-#1, TUV-R4D-#2 and TUV-R4D-#4 are the amino acids which the displayed peptide sequences have in common.

5 3/6 clones were unique (#3, #5, and #6) clones.

Amino Acid Sequence displayed by clone TUV-R4D-#3:

Gly Cys Gly Gly Phe His Pro Gly Ser Asn Cys Leu Val Val Ser
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

10

This peptide is identical to clone YCA-4R4-#10 in Example 3 below. Thus, TUV-R4D-#3 = YCA-4R4-#10.

15 TUV-R4D-#3 was also identical to six of the clones selected at R5D, to two of the clones selected at R5E and to seven clones selected at R5H.

In addition, ten clones were sequenced after R5D, i.e. the tissue eluted fraction from umbilical vein after round 5:

20

6/10 clones were identical to clone TUV-R4D-#3.

4/10 identical clones were designated TUV-R5D-#2.

25 Amino Acid Sequence displayed by clone TUV-R5D-#2

Arg Asn Val Pro Pro Ile Phe Asn Asp Val Tyr Trp Ile Ala Phe
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

30 The underlined sequence appears in clone TUV-R4B*-#3 as well. Furthermore, this clone is identical to clone YCA-5R1-#11 (Example 3). Thus, TUV-R5D-#2 = YCA-5R1-#11

WO 98/39469

PCT/US98/04188

-36-

Table 8: Biopanning of umbilical artery with a mixture of the 15-mer and 6-mer libraries (4.5×10^9 TTU each) - yield of tissue elution using Protocol N1 (Experiment E)

5	Round #	Input (TTU)	Output (TTU)	Yield (%)
	R1E	9×10^9	3×10^6	3×10^{-3}
	R2E	7×10^{10}	2×10^5	2×10^{-4}
	R3E	2×10^{10}	1×10^7	6×10^{-2}
	R4E	1×10^{11}	4×10^6	4×10^{-3}
10	R5E	5×10^{11}	3×10^8	6×10^{-2}

Ten clones were sequenced after R5E, i.e. the tissue eluted fraction from umbilical artery after round 5:

15 3/10 clones were identical to TUV-R4D-#3, which is also identical to YCA-4R4-#10 (Example 3).

1/10 clone was identical to TUV-R4B*-#3.

20 6/10 clones were unique clones.

WO 98/39469

PCT/US98/04188

-37-

Table 9: Biopanning of umbilical artery with 15-mer library
- yield of tissue elution using Protocol R2 (Experiment F).

Round #	Input (TTU)	Output (TTU)	Yield (%)
R1F	5×10^8	4×10^6	7×10^{-1}
R2F	2×10^{10}	6×10^5	3×10^{-3}
R3F	2×10^{10}	2×10^5	1×10^{-3}
R4F	2×10^{10}	1×10^6	6×10^{-3}
R5F	9×10^9	4×10^7	5×10^{-2}

Ten clones were sequenced after R5F, i.e. the tissue eluted fraction from umbilical artery after round 5:

8/10 clones were identical to clone TUV-R4B*--#1.

1/10 clone was identical to clone TUV-R4B*--#3 and 1/10 clone was a unique clone.

WO 98/39469

PCT/US98/04188

-38-

Table 10: Biopanning of 15-mer library with umbilical artery with vein-excluded¹ phage library - yield of tissue elution using protocol N1 (Experiment H).

	Input (TTU)	Output (TTU)	Yield (%)
R1H	1×10^9	2×10^5	2×10^{-2}
R2H	3×10^9	8×10^6	3×10^{-1}
R3H	6×10^9	6×10^7	9×10^{-1}
R4H	-	8×10^6	-
R5H	8×10^{10}	3×10^7	3×10^{-2}

¹ Phage library was first biopanned on the umbilical vein (8-10cm length), and only the unbound (vein-excluded) phage was then biopanned with umbilical artery (4-5 cm length). This sequence was repeated for all rounds. Tissue elution with starved bacteria was conducted according to protocol N1.

Seven clones were sequenced after R5H, i.e. the tissue eluted fraction from umbilical artery after round 5: 7/7 clones were identical to clone TUV-R4D-#3.

WO 98/39469

PCT/US98/04188

-39-

Table 11: Binding of clone TUV-R4B*--#1 and clone TUV-R4B*--#3 (see section 3 below) to endothelial cell culture (Protocol EC-1); results of acid, tissue and urea elutions.

	Total colonies	clone TUV-R4B*--#1 (TTU)	clone TUV-R4B*--#1 (%)	clone TUV-R4B*--#3 (TTU)	clone TUV-R4B*--#3 (%)
input	2×10^9	3.4×10^7	1.7	1.2×10^7	0.6
acid elution	5×10^5	7×10^3	1.4	5×10^3	1.0
tissue elution	3×10^4	5.7×10^2	1.9	N.D. ¹	N.D.
urea elution	5×10^4	5×10^4	100	N.D.	N.D.

¹ N.D. = not detectable.

The results indicate that clone TUV-R4B*--#1 binds strongly to endothelial cells or to endothelial cell extra-cellular matrix, since 100% of the phages eluted with urea were identical to clone TUV-R4B*--#1.

3. Tissue distribution of TUV-R4B*--#1

In order to analyze tissue specificity of the selected phage displayed peptides, a phage mixture (4×10^{10}) of a selected clone, either clone TUV-R4B*--#1 or clone TUV-R4B*--#3 or both clones together, with the 15-mer library (in a ratio of 1:10- 1:100) was injected through the tail vein of a rat (in 0.5ml DMEM-1% BSA). After 4 minutes, the animal was sacrificed, the chest was opened, and extensive washing of the blood vessels was carried out by flushing isotonic salt solution through the left ventricle. After approximately 10 minutes, as the lungs became discolored, various organs were dissected, weighed, and homogenized in 1ml homogenization solution (DMEM-1%BSA-Pi) using an ultra thorax grinding device. Following centrifugation (10 minutes at top speed eppendorf centrifuge), the supernatant was discarded, and

WO 98/39469

PCT/US98/04188

-40-

the pelleted tissue was washed three times with the same buffer. The washed tissue extract was resuspended in 0.6ml NZY and 0.4 ml starved bacteria (2×10^{10}) and was incubated with gentle mixing for 10 minutes at 37°C. The tissue-bacterial suspension was diluted in 10ml NZY containing 0.2% tetracycline and after 45 minutes of vigorous mixing at 37°C, aliquots were plated on agar plates containing 40µg/ml tetracycline- 20µg/ml kanamycin and incubated at 37°C for 16 hours. After monitoring tet^R colonies on each plate, the colonies were transferred to a millipore sheet for colony hybridization as described in "Molecular Cloning: A Laboratory Manual", J, Sambrook, E.S. Fritsch, and T. Maniatis, Cold Spring Harbor Laboratory Press, 2nd edition, 1989.

Synthetic DNA probes of 24 and 23 oligonucleotides derived from the antisense sequence of clones TUV-R4B*-#1 and TUV-R4B*-#3 respectively, were used.

WO 98/39469

PCT/US98/04188

-41-

Table 12: Tissue distribution of TUV-R4B*--#1 in a rat model (the ratio of clone TUV-R4B*--#1 to 15-mer library in the input phage was approximately 1:10).

5

10

	¹ Total No. of colonies	² No. of TUV- R4B*--#1	% of TUV- R4B*--#1	% of 15-mer library
Input	167	15	8.9	91.1
Lung	145	144	99.3	0.7
Vena Cava ³	70	47	67.1	32.9
Aorta ⁴	110	75	68.2	31.8
Liver	174	110	63.2	36.8
Spleen	108	58	53.7	46.3
Brain	204	130	63.7	36.3
Kidney	198	135	68.1	31.9

15

¹ Total bacterial colonies on agar plates.

² Positive colonies after hybridization to the antisense radioactive probe of TUV-R4B*--#1.

20

³ Example of Vein.

⁴ Example of Artery.

WO 98/39469

PCT/US98/04188

-42-

Tables 13A and 13B: Tissue distribution of clone TUV-R4B*--#1 and TUV-R4B*--#3 in rat model

The ratio of TUV-R4B*--#1 and TUV-R4B*--#3 to the 15-mer library was approximately 1:1:98.

5

Table 13A: Tissue distribution of TUV-R4B*--#1

	¹ Total No. of colonies	² No. of TUV- R4B*--#1	% of TUV- R4B*--#1	% of 15-mer library
Input	800	16	1.1	98.9
Lung	9	8	88.8	11.2
Vena Cava	110	47	42.7	57.3
Aorta	249	106	42.5	57.5
Liver	230	152	66.0	33.0
Spleen	54	37	68.5	31.5
Brain	23	8	34.7	65.3
Kidney	22	12	54.5	45.5

¹ Total bacterial colonies on agar plates.

² Positive colonies obtained by hybridization to the antisense radioactive probe of TUV-R4B*--#1.

The results of tables 12 and 13A demonstrate that clone TUV-R4B*--#1 is enriched in the rat lung, i.e. has highest specificity to the lung. Therefore, this peptide can be used as a specific marker for *inter alia* lung tissue, for drug delivery to lung tissue and to image lung tissue.

WO 98/39469

PCT/US98/04188

-43-

Table 13B: tissue distribution of clone TUV-R4B*--#3

	¹ Total No. of colonies	² No. of clone TUV- R4B*--#3	% of TUV- R4B*--#3	% of 15-mer library
Input	484	4	0.82	99.1
5 Lung	60	10	16.6	83.4
Vena Cava	53	31	5.8	94.2
Aorta	416	41	9.8	90.2
Liver	204	59	28.9	71.1
Spleen	38	7	18.4	81.6
10 Brain	29	5	17.2	82.8
Kidney	37	8	21.6	78.4

¹ Total bacterial colonies on agar plates.

15 ² Positive colonies by hybridization to the antisense
radioactive probe of TUV-R4B*--#3.

-44-

EXAMPLE 2: Isolation and Selection of Peptide Epitopes which Specifically Bind to Undetermined Targets in Safenal Vein, Umbilical Artery and Radial Artery.

- 5 As described in Example 1, a 6-mer phage display peptide library was kindly provided by G. Smith (Virology 167: 156-165, 1988). The library was amplified to form a phage library working stock.
- 10 The following protocol was used for the identification of peptides that specifically bind to
- (1) safenal vein (JSV1);
 - (2) umbilical artery (JUA1); and
 - 15 (3) radial artery (JRA1).

The 6-mer bacteriophage library displayed on the pIII coat protein was used, and the selection procedure was carried out by perfusion for 4 or 5 rounds of biopanning essentially
20 as described for Protocol R1 and R2 in Example 1.

Before panning with the phage library, blood vessels were prewashed by perfusing cold (4°C) DMEM-5% human serum/5U/ml heparin solution.

25 Following ex-vivo perfusion of the phage library (in the first round approximately 2×10^{12} phages were used), non-specifically bound phages were extensively washed with DMEM 5% human serum containing a mixture of protease inhibitors (Pi). Tightly bound phages were eluted at room temperature
30 sequentially with 2ml trypsin 0.25%/EDTA 0.05% solution and then with 5ml acid (0.2M glycine-HCl pH-2.2) solution, containing 0.5% Tween (Protocol R1). The recovered phages from both washes were combined, neutralized, titered and
35 amplified. Although the above treatment was sufficient to recover most of the bound phage, some phages remained associated with tissue. This tissue associated fraction was also recovered by displacement with prestarved *E. coli* cells

WO 98/39469

PCT/US98/04188

-45-

perfused into the acid washed blood vessels at 37°C for 1 hour (Protocol R2). The tissue fraction was titrated and amplified as a separate stock for each blood vessel [tissue wash safenal vein (JTSV1), tissue wash umbilical artery (JTUA1) and tissue wash radial artery (JTRA1), respectively].

In the subsequent rounds of phage amplification, the experiments were carried out essentially as described above, except that approximately 2×10^{10} phages were used for each cycle of biopanning, while maintaining tissue and fraction specificity (for example, when an acid-eluted fraction of the phage was initially recovered (R1), then only the acid fraction was amplified in all subsequent rounds).

After four to five rounds of biopanning there was an enrichment in the recovered phages by at least 3 orders of magnitude compared to the yield obtained after the first round of panning.

To examine the effect of the above treatments (including the wash with trypsin/acid/tween as described in protocol R1 in Example 1) on the endothelial cell layer which line the inner side of the vessel wall, histological sections were prepared. No major changes were observed in the endothelial cells surrounding the lumen of the perfused blood vessels compared to non-treated vein or artery.

After the selection and enrichment procedure, single stranded DNA was prepared for sequencing from individual colonies of phage-infected bacteria. After five rounds of amplification of the safenal vein acid elution phage fraction, twenty-two clones were sequenced. Apart from one peptide sequence designated JSV1-#13, which appeared twice (9%), none of the phage peptide sequences were identical.

Amino acid sequence displayed by clone JSV1-#13

Phe Leu Pro Asn Gly Phe

WO 98/39469

PCT/US98/04188

-46-

1 2 3 4 5 6

In contrast, after four rounds of amplification, five of seven (71%) safenal tissue associated phages were identical. The peptide encoded by these phages was designated JTSV1-#7. The other two clones designated JTSV1-#26 were also identical.

Amino acid sequence displayed by clone JTSV1-#7

10 Thr His Asp Thr His Leu

1 2 3 4 5 6

Amino acid sequence displayed by clone JTSV1-#26

Thr His Glu Thr Gln Arg

15 1 2 3 4 5 6

All six clones isolated after umbilical artery selection from the acid elution fraction were different. One of these peptides (16%), was designated JUA1-#1. This amino acid sequence was also isolated once (1/7, 14%) from the tissue associated umbilical artery (JTUA1) phage stock and once (1/22, 4.5%) from the tissue associated safenal vein (JTSV1) phage stock.

25 Amino acid sequence displayed by clone JUA1-#1

Asp Ile Ala Lys Arg Tyr

1 2 3 4 5 6

30 Interestingly, after four rounds of biopanning, the amino acid sequence of JTSV1-#7, was also recovered from JTUA1 (tissue associated umbilical artery) amplified phage stock with an identical frequency (5/7, 71%) as from JTSV1 (tissue associated fraction). Moreover, seven of seven clones (100%) of JTRA1 enriched fraction were identical to JTSV1-#7. The other two clones in the JTUA1 tissue associated fraction were different. This result suggests that clone JTSV1-#7 binds specifically to a general target in the blood vessel, regardless of its source.

WO 98/39469

PCT/US98/04188

-47-

In an attempt to eliminate the binding of JTSV1-#7 to artery, the 6-mer library (5×10^{10} TTU) was first passed through the umbilical vein and the unbound phages were passed directly through the umbilical artery. The protocol for the biopanning, washing and elution was basically as described for the tissue associated fraction in the first set of experiments (for example JTUA1)

Following four rounds of biopanning, the tissue associated amplified phage stock was designated JTUVA2. Single stranded DNA was prepared from fourteen random clones. Thirteen of fourteen (13/14, 93%) clones isolated from the JTUVA2 phage stock were identical to clone JTSV1-#7.

Organ and cell binding specificities of clones JTSV1-#7, JSV1-#13 and JTSV1-#26 were each evaluated by biopanning separately in a mixture with the 6-mer initial stock library. The phage binding specificity to several organs removed from mice injected into the tail vein with phage mixture is shown in Table 1. Binding to human blood vessels perfused ex vivo is shown in Table 2.

The specific phage binding frequency was detected by hybridization with a radioactive antisense oligonucleotide complementary to the sequence encoded by the corresponding phage.

WO 98/39469

PCT/US98/04188

-48-

TABLE 1

5 Tissue distribution of clones JTSV1-#7, JTSV1-#26 and JSV1-
#13 in mouse model. The input was 0.1% of each clone within
10¹⁰TTU 6-mer library).

	JTSV1-#7	JSV1-#13	JTSV1-#26
Heart	Binds at	3%	24%
Spleen	a high	1%	2%
Brain	frequency	3%	0%
10 Lung	to all	5%	0%
Kidney	organs	12%	0%
Liver		1%	0%

WO 98/39469

PCT/US98/04188

-49-

TABLE 2

Binding specificity of clones (5% each within 10^{10} 6-mer library) to umbilical cord (artery or vein)

5

Input clone (5%)	Umbilical Artery	Umbilical Vein	Mammary Artery	Safenal Vein
JTSV1-#7	>80%	>80%	-	-
JSV1-#13	13%	25%	-	-
JTSV1-#26	20%	80%	-	-
10 (Mixture of - Hybridization with JSV1-#13	0.1%	28%	10-20%	0.1%
15 JSV1-#13 and JTSV1-#26 - Hybridization with JTSV1-#26	0.2%	50%	0.1%	20%

The specific hybridization assays show that clone JTSV1-#26 is vein specific, whereas clones JTSV1-#7 and JSV1-#13 bind vein and artery at about the same affinity (see Table 2).

-50-

EXAMPLE 3: Isolation of peptides which specifically bind to undetermined targets in rat coronary artery

Peptide display phage library source

5 As described in Example 1, a 15-mer phage display peptide library was kindly provided by G. Smith (Virology 167: 156-165, 1988). The library was amplified to form a phage library working stock.

10 **Preparation of perfused rat coronary arteries**

Isolated rat hearts were subjected to a Langendorff-type perfusion system, as described by Neely & Rovetto (1975), Methods in Enzymology 39: 43-60), using a modified Krebs-Henzeleit bicarbonate buffer, pH 7.4, equilibrated with
15 O₂:CO₂(95:5) at 37°C as the basic perfusion medium. The system consisted of a 90 cm glass jacketed condenser, in which a constant 90 cm head of the medium was kept (by use of an overflow system). The temperature was kept constant by a flow of thermostated water through the jacket, and
20 equilibration with the gas mixture was assured by slow bubbling of the gas at the bottom of the inner section of the condenser, facilitated through a narrow polyethylene tube inserted from the top. A three-way stopcock was connected between the outlet of the condenser and the heart
25 cannula (made of a 18-gage needle), and an infusion pump was connected to the side arm of the stopcock using a narrow polyethylene tubing (thus keeping a minimal dead volume).

After connection of the organ to the cannula, the buffer was
30 allowed to flow freely into the heart, thus facilitating regular beating. The flow rate (7-12ml/min) was then measured, followed by an initiation of a sidearm flow of 5% BSA in the same buffer at a 1/9th rate, thus resulting in a final concentration of BSA of 0.5% in the heart perfusate.
35 The hearts were perfused at these conditions for 10 min. The three-way valve was then adjusted to flow through the sidearm only, and a suspension of the phages (10¹⁰-10¹² phages) in 20 ml of 0.5% BSA in perfusion buffer was

WO 98/39469

PCT/US98/04188

-51-

introduced into the heart at a flow rate of 4ml/min. The free flow of perfusion buffer + BSA 0.5% was resumed for an additional 10 minutes. At the end of the wash period, the valve was again adjusted to a sidearm flow only, and 0.1 ml of a solution of Trypan Blue (% in) was injected into the heart vasculature, in order to clearly visualize the coronary arteries. The heart was then removed, and a piece of tissue, consisting mainly of the coronary artery, was excised and taken for homogenization.

10

Bacterial strain

The bacterial strain used was as described above in Example 1.

15

Biopanning protocols

Several biopanning protocols were developed:

20

A . Protocol YMCA-1

B. Protocol YMCA-2

C. Protocol YMCA-4

D. Protocol YMCA-5

Protocol YMCA-1

1. Prewash: the heart vasculature was washed in Krebs-Henzeleit bicarbonate buffer (perfusion buffer), 0.5%BSA through the aorta for 10 minutes, at a flow rate of 8-12 ml/min.

25

2. Ex vivo selection was carried out at 10^{10} - 2×10^{11} TTU in 20 ml perfusion buffer, 0.5% BSA-Pi at a flow rate of 4 ml/min for 5 min at 37°C.

30

3. Wash was carried out in perfusion buffer, 0.5%BSA for 10 min at 8-12 ml/min.

35

4. Elution: The coronary artery was excised and gently grounded in 0.3 ml DMEM, 0.5%BSA-Pi. The tissue was washed

WO 98/39469

PCT/US98/04188

-52-

with 0.3 ml DMEM, 0.5%BSA-Pi. Tissue-associated phages were recovered at room temperature during 1 hour, by displacement with 2×10^9 starved *E.coli* cells in 0.3 ml.

5 TABLE 1: Biopanning of 15-mer library in rat coronary artery (protocol YMCA-1)

BIOPANNING ROUND	INPUT	OUTPUT	TISSUE- ASSOCIATED PHAGE (%)
10 YMCA-1, R-1	10^{10}	6×10^4	6×10^{-4}
YMCA-1, R-2	2×10^{11}	8×10^4	4×10^{-5}
YMCA-1, R-3	2×10^{10}	1.2×10^5	6×10^{-4}
YMCA-1, R-4	10^{11}	9.6×10^5	10^{-3}
15 YMCA-1, R-5	2×10^{11}	3.6×10^7	1.2×10^{-2}

The results of this experiment demonstrated a 100 fold increase of tissue-associated phage between the first and fifth round (R) of biopanning.

20 The DNA sequence of seven independent tissue-associated phages was determined after round 5. Three of these clones were designated YCA-1R5-#11, YCA-1R5-#2 and YCA-1R5-#13.

Amino acid sequence displayed by clone YCA-1R5-#11

25 Arg Asn Val Pro Pro Ile Phe Asn Asp Val Tyr Trp Ile Ala Phe
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Clone YCA-1R5-#11 is identical to clone TUV-R5D-#2 selected independently from umbilical vein in Example 1.

30

Amino acid sequence displayed by clone YCA-1R5-#2

Asp Val Ser Pro Val Trp Ala Ala Phe Ala Ser Gly Ala Ser Phe
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

35

Amino acid sequence displayed by clone YCA-1R5-#13

Ser Leu Trp Gly Ala Ser Ser Cys Gly Val Ala Phe Phe Glu Ser

WO 98/39469

PCT/US98/04188

-53-

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Protocol YMCA-2

1. Prewash was carried out as in protocol YMCA-1.

5

2. Selection was carried out as in protocol YMCA-1

3. Wash was carried out as in protocol YMCA-1

10 4. Elution: the coronary artery was excised and gently
grounded by homogenizer (KIKI-WERK) (1000 rpm) in 0.3 ml
DMEM, 0.5%BSA-Pi. The tissue was washed in 0.3ml 0.13M
glycine-HCl, pH2.2 for 5 minutes, neutralized in 21µl Trisma
base, washed in 0.3ml DMEM, 0.5%BSA-Pi and tissue-associated
15 phages were recovered at room temperature for 1 hour by
displacement with 2×10^9 starved E.coli cells in 0.3 ml.

WO 98/39469

PCT/US98/04188

- 54 -

TABLE 2: Biopanning of 15-mer library with rat coronary artery (protocol YMCA-2)

BIOPANNING ROUND	INPUT	OUTPUT	TISSUE- ASSOCIATED PHAGE (%)
YMCA-2, R-1	10^{10}	4.6×10^4	4.6×10^{-4}
YMCA-2, R-2	5×10^{11}	2.5×10^5	5×10^{-5}
YMCA-2, R-3	10^{10}	1.6×10^4	1.6×10^{-4}
YMCA-2, R-4	10^{11}	10^5	1×10^{-4}
YMCA-2, R-5	10^{11}	10^5	1×10^{-4}

7/7 clones from round 5 were identical to clone YCA-4R4-#10 (see YMCA-4 below).

15 Protocol YMCA-4

1. Prewash was carried out as in protocol YMCA-1.

2. Selection was carried out as in protocol YMCA-1

20 3. Wash was carried out as in protocol YMCA-1

4. Elution: the coronary artery was excised, washed in 0.3ml 0.13M glycine-HCl, pH2.2 for 5 minutes and neutralized with 21μl Trisma base. The tissue was then ground vigorously in 0.3 ml DMEM, 0.5%BSA-Pi and tissue-associated phages were recovered at room temperature for 1 hour by displacement with 2×10^9 prestarved *E.coli* cells in 0.3 ml.

TABLE 3: Biopanning of 15-mer library with rat coronary artery (protocol YMCA-4)

BIOPANNING ROUND	INPUT	OUTPUT	TISSUE-ASSOCIATED PHAGE (%)
YMCA-4, R-1	10^{10}	2.2×10^5	2.2×10^{-3}
YMCA-4, R-1	10^{10}	8×10^5	8×10^{-3}
YMCA-4, R-2*	3×10^{10}	1×10^6	3×10^{-3}
YCA-4, R-3	10^{11}	2.4×10^6	2.4×10^{-3}
YMCA-4, R-4	10^{11}	1.4×10^7	1.4×10^{-2}
YMCA-4, R-5	2×10^{11}	3.8×10^7	1.9×10^{-2}

* The input phage of R-2 was a mixture of the output of both R-1's.

The results of this experiment demonstrated a 100 fold increase between the first and the fourth round of panning. There was no significant increase between the fourth and fifth round of panning.

Ten clones were sequenced after the fourth round:

(i) 4/10 (40%) clones were identical (clones 6,7,9,10) to clone YCA-4R4-#10 (see YMCA-2).

Amino acid sequence displayed by clone YCA-4R4-#10:

Gly Cys Gly Gly Phe His Pro Gly Ser Asn Cys Leu Val Val Ser
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Because of the cysteines in positions 2 and 11, this clone may be a cyclic peptide. This sequence is positively charged (+1).

In addition, a similar sequence to Gly Cys Gly Gly (underlined) was found in clone YCA-4R4-#3 (see below) which comprises the sequence Gly Leu Gly Gly. Therefore, Gly X Gly Gly may be a consensus binding sequence, wherein X is

WO 98/39469

PCT/US98/04188

-56-

Cys or Leu or any other amino acid.

Moreover, the sequence Asn Cys Leu (underlined) also appeared in clone YCA-4R4-#5 (see below) and thus, this sequence may be a consensus binding sequence.

Furthermore, clone YCA-4R4-#10 is identical to clone TUV-R4D-#3 selected independently in veins of human umbilical cord, as disclosed in Example 1.

- (ii) 2/10 (20%) clones were identical to YCA-1R5-#2.
- (iii) 4/10 (40%) clones were unique. These clones were designated YCA-4R4-#5, YCA-4R4-#4, YCA-4R4-#3 and YCA-4R4-#8.

Amino acid sequence displayed by clone YCA-4R4-#5

Ser Asp Cys Leu His Ser Val Arg Gly Phe Asn Cys Leu Lys Arg
 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Clone YCA-4R4-#5, although a unique clone, is interesting because it has the motif Asn Cys Leu which was also found in clone YCA-4R4-#10. Furthermore, clone YCA-4R4-#5 has cysteine residues in positions 3 and 12 and thus the displayed peptide may be cyclic. Clone YCA-4R4-#5 has a positive charge of +3.

Amino acid sequence displayed by clone YCA-4R4-#4

Ser Leu Cys Phe Tyr Leu Phe Val Met Ser Ala Pro Asp Ala Pro
 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Amino acid sequence displayed by clone YCA-4R4-#3

Gly Leu Gly Gly Leu Ser Phe Gly His Ser Asp Asn Pro Pro Ser
 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

The sequence Gly Leu Gly Gly (underlined) is of interest because a similar sequence appeared in clone YCA-4R4-#10 as discussed above.

WO 98/39469

PCT/US98/04188

-57-

Amino acid sequence displayed by clone YCA-4R4-#8

5 Gly Pro Gly Trp Val Gly Trp Phe Val Ser Leu Phe Tyr Ala Ser
 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

In addition, seven clones were sequenced after the fifth round. One of these clones was designated YCA-4R5-#12.

10 **Amino acid sequence displayed by clone YCA-4R5-#12:**

 Trp Ser Leu Gly Ser Ser Trp Val Tyr Lys Phe Phe Tyr Ser Ser
 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Protocol YMCA-5 - In vivo selection

- 15 To select peptides in vivo that specifically bind to the coronary artery, 2×10^{11} TTU of amplified phage eluate from YMCA-4, R-4 diluted in 0.5ml DMEM from YMCA-4, R-4 was injected intravenously to live rats (into the tail vein). After 5 minutes animals were anesthetized and subsequently
- 20 sacrificed. The heart was removed and washed with perfusion buffer 0.5% BSA for 10 minutes at 8-12 ml/min. The coronary artery was isolated, excised and the bound phages were eluted as described above for protocol YMCA-4.
- 25 Phages were recovered from the coronary artery, titered, amplified and sequenced.

WO 98/39469

PCT/US98/04188

-58-

TABLE 4: *In vivo* biopanning on rat coronary artery (protocol YMCA-5)

BIOPANNING ROUND	INPUT	OUTPUT	TISSUE- ASSOCIATED PHAGE (%)
YMCA-5, R- 1	2×10^{11}	5×10^5	2.4×10^{-4}

DNA of seventeen clones were sequenced, three of which were identical to YCA-1R5-#11.

This experiment was performed together with YMCA-4, R-5 as a control. The input in both experiments was amplified eluate YMCA-4, R-4, 2×10^{11} TTU.

Tables 3 and 4 demonstrate that the output of clone YMCA-5, R-1 was 100 fold less (5×10^5) then the output of clone YMCA-4, R-5 (3.8×10^7).

-59-

EXAMPLE 4: Preparation of a peptide

A peptide of the subject invention, corresponding to the sequence of a peptide displayed by a phage virion, is prepared by the following methods:

I. Chemical (organic) synthesis

Chemical synthesis of peptides is carried out by methods well-known in the art, e.g. solid phase synthesis of Merrifield, J. Amer. Chem Soc. 85: 2149-2154 (1963); Science 150: 178-185 (1965); Ibid 232, 341-347 (1986). The solid phase synthesis provides a growing peptide chain anchored by its carboxyl terminus to a solid support, e.g. a resin such as chloromethylated polystyrene resin or p-methylbenzhydrylamin resin when synthesizing a peptide amide derivative. The use of various N-protecting groups, e.g. the carbobenzyloxy group, the t-butyloxycarbonyl group (BOC) or the N-(9-fluorenyl-methylcarbonyl) group (Fmoc), various coupling reagents, e.g. dicyclohexylcarbodiimide (DCC) or carbonyldiimidazole, various cleavage reagents, e.g. trifluoroacetic acid (TFA) in methylene chloride (CH_2Cl_2) and other such reagents of classical solution phase peptide synthesis are also used in conventional solid phase synthesis of peptides.

Various commercial companies prepare peptides custom-made, i.e. specifically as ordered by customers. Examples of such companies are Bio-Synthesis, Research Genetics, Inc., and AnaSpec (Science 275: 270 (1997)).

The chemically synthesized peptide is linked to a pharmaceutical agent (drug) *inter alia* by a covalent or by a non-covalent bond forming a drug-peptide conjugate. The peptide may also be incorporated into a liposome. The covalent bond may be *inter alia* a peptide, an amide, an ester, a disulfide or an anhydride covalent bond. The non covalent bond is *inter alia* an ionic bond or a hydrophobic complex. The pharmaceutical agent may be *inter alia* a radio

WO 98/39469

PCT/US98/04188

-60-

isotope label.

The choice of the peptide linkage is determined based on the functional groups of the individual drug.

5

The peptide may also be cyclic (see e.g. Example 3). Cyclic peptides having a disulfide bond may be prepared as described in U.S. Patent No. 4,903,773 (Partoliano and Ladner).

10

II. Recombinant technology

Recombinant production of peptides is carried out by methods known in the art. The DNA encoding the peptide is prepared by synthetic oligonucleotides based on the amino acid sequence of the peptide and their known nucleotide codons (see e.g. U.S. Patent No. 5,221,619). The peptide is then produced by expression of the nucleotide sequence encoding the polypeptide.

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The recombinantly produced peptide is linked to a pharmaceutical agent. When the pharmaceutical agent is a recombinant polypeptide, a hybrid fusion polypeptide is constructed comprising the peptide linked by a peptide bond to the drug as follows: a DNA molecule is prepared comprising DNA encoding the drug and DNA encoding the peptide. The DNA encoding the peptide is prepared by synthetic oligonucleotides based on the amino acid sequence of the peptide and their known nucleotide codons. The synthetic oligonucleotide encoding the peptide is ligated either to the 5' end of the DNA strand encoding the drug or to the 3' end. When ligated to the 5' end of the DNA encoding the drug, an ATG nucleotide sequence is added to the synthetic oligonucleotide encoding the peptide. When ligated at the 3' end of the DNA encoding the drug, a DNA termination codon (TAA or TGA) is added at the 3' end of the synthetic oligonucleotide encoding the peptide and the termination codon at the 3' end of the DNA encoding the drug is removed using conventional recombinant DNA technology

-61-

methods by use of Mung bean nuclease or cleavage with an appropriate restriction enzyme.

5 The hybrid polypeptide encoded by the above described recombinant DNA molecule is expressed and produced by recombinant technology by methods known in the art, e.g. in bacteria, yeast, insect, plant or mammalian cells in culture or in a genetically engineered transgenic animal or plant.

10 III. Computer-aided design technology

In recent years, intensive efforts have been made to develop peptidomimetics or peptide analogs that display more favorable pharmacological properties than their prototype native peptides. The native peptide itself, the
15 pharmacological properties of which have been optimized, generally serves as a lead for the development of these peptidomimetics. A lead structure for development of a peptidomimetic can be optimized, for example, by molecular modeling programs.

20

Computer-aided design technology known in the art is used in order to produce a mimotope, i.e. a peptide mimetic of a peptide of the subject invention, the chemical structure of which is different from the peptide, but the biological
25 activity of which remains similar to that of the peptide. U.S. Patent Nos. 4,879,313, 4,992,463 and 5,0191,396 describe examples of such peptide mimetic compounds.

To a peptide of the subject invention, produced by any one
30 of the above three methods, amino acid residues may be added, deleted or substituted using established well known procedures, thereby producing extended peptides.

Furthermore, the DNA encoding a peptide of the subject
35 invention may be mutated by methods known to those skilled in the art, thereby produced mutated peptides.

As mentioned above, a peptide of the subject invention,

WO 98/39469

PCT/US98/04188

-62-

produced by any one of the above three methods, may be administered to a patient, alone, radiolabeled, or linked to a pharmaceutical agent (drug).

5 The following pharmaceutical agents are examples of drugs which are linked to a peptide of the subject invention corresponding to a peptide displayed by a phage virion to form drug-peptide conjugates. Other pharmaceutical agents not mentioned below may also be used.

10 Peptides of the subject invention are linked to a toxin, an anti-cancer drug or an anti-angiogenic compound in order to target and destroy tumor tissue. This specific use of such drug-peptide conjugates represents an alternative approach to current attempts to apply immunotoxins against cancer.

15 Solid tumor growth *in vivo* is associated with recruitment of new blood vessels. Targeting the tumor-vasculature is an attractive possibility for anti-cancer therapy for the following reasons: First, most anti-cancer drugs are given

20 by the systemic intravenous route and drug concentration at the tumor tissue is the crucial factor for effective therapy. Secondly, one limitation of current anti-cancer therapy is toxicity. Targeting the drug by cross-linking with a tissue specific peptide circumvents the toxicity

25 problem. Thirdly, targeted anti-vascular or anti-angiogenesis therapy obliterates the capacity of the tumor to continue to grow and metastize as blood supply of fresh nutrient is blocked.

30 Examples of anti-cancer drugs that are linked to the peptides of the subject invention are adriamycin, cis-Platinum, taxol, bleomycin and so forth.

35 Examples of anti-angiogenic compounds that are linked to peptides of the subject invention are cortisone, heparin and so forth.

Examples of toxins that are linked to peptides of the

-63-

subject invention are *Pseudomonas* exotoxin A, ricin and so forth.

5 Several major drugs applied in heart conditions are given by the systemic intravenous route while their target, the coronary arteries, constitute only a small portion of the vasculature. Therefore, targeting to the coronaries ,or even to arteries in general concentrates the drug to the diseased vessels and reduces undesired side effects.

10 Examples of cardiovascular drugs that are linked to the peptides of the subject invention are thrombolytic enzymes such as tissue plasminogen activator (tPA), streptokinase (SK), and anti-thrombotic agents such as heparin,
15 ticlopidine or antiplatelet monoclonal antibodies.

Peptides of the subject invention are further linked to recombinant proteins such as CuZnSOD, MnSOD, Factor Xa Inhibitors, erythropoietin, von Willebrand Factor or
20 fragments thereof, ecto-enzymes such as Apyrase and so forth. Such recombinant proteins are described *inter alia* in U.S. Patent Nos. 5,126,252 and 5,360,729 (CuZnSOD), U.S. Patent Nos. 5,270,195 and 5,246,847 (MnSOD), WO 91/01416 (von Willebrand factor fragment), WO 94/03871 (Factor Xa
25 Inhibitor), and U.S. Patent No. 4,703,008 (erythropoietin).

The most common brain disorders include tumors, stroke, head trauma, epilepsy, infectious agents, Parkinson's disease and Alzheimer's disease. Several drugs have been approved for
30 these indications. However, there is a need to increase the level of drug reaching the brain vasculature and developing a delivery system which transports therapeutics across the blood-brain barrier (BBB).

35 Examples of "CNS drugs" that are linked to the peptides of the subject invention are L-Dopa, Cortisone, tPA or phenobarbital.

WO 98/39469

PCT/US98/04188

-64-

Both the liver and the kidney are suitable for targeted drug delivery via the vascular system since they are well perfused tissues, involved in the metabolism of many endogenous and exogenous compounds. Targeting of liver and kidney is important particularly for cancer therapy and hepatitis B and C virus infection of the liver.

Examples of drugs for treatment of liver and kidney disease that are linked to peptides of the subject invention are anti-viral drugs such as Interferon, Iododeoxyuridine, and Adenine arabinoside. Adriamycin can be used for the treatment of renal cancer and Glucocerebrosidase 6-thioguanine for the treatment of Gaucher's disease.

Upon injection, a recombinant protein is targeted to tissue specific endothelial cells and is converted from a soluble protein to a cell surface bound protein (ectoenzyme). In this manner, arteriosclerosis and thrombogenicity of the vessel wall in certain clinical indications is reduced. Conversion of these soluble proteins into ectoenzymes also reduces blood clearance time and hence a lower amount of recombinant protein is injected to achieve efficacy.

-65-

EXAMPLE 5: Selection of Tissue-Specific Epitopes which Preferentially Bind to a Damaged Artery

5 Biopanning of a damaged human artery with a phage peptide library.

Local damage is made in an artery by using the current methodology of angiography, PTCA (percutaneous transluminal coronary angiography) ballooning. Upon insertion of the inflated balloon, denudation of blood vessel endothelial cells occurs and the subendothelium is exposed. The exposed subendothelium is thrombogenic and causes post PTCA reocclusion and restenosis.

15 Displayed peptides having specific amino acid sequences are obtained by biopanning the damaged artery with a phage display peptide library. Phages that specifically bind the exposed subendothelium (but not the normal ("healthy") blood vessel endothelial cells) are isolated and the DNA sequence is determined in order to obtain the corresponding amino acid sequence of the displayed peptide.

25 The peptide corresponding to the displayed peptide sequence (or a peptide mimetic thereof) is linked to an anti-coagulant and/or anti-antithrombotic agent and/or anti-arteriosclerotic agent as described in Example 4. The peptide or peptide-drug conjugate is used to target exposed subendothelium and prevent platelet aggregation, thrombus formation, restenosis and arteriosclerosis.

WO 98/39469

PCT/US98/04188

-66-

EXAMPLE 6: Isolation of tumor specific endothelium epitopes by ex vivo perfusion of a phage display library through a solid tumor vascular system derived from human colon, lung, kidney, liver and ovary carcinoma tissues

5

Human colon, lung, kidney, liver and ovary carcinoma tissues obtained after surgery are each separately perfused ex vivo via their vascular system, and biopanned with a phage peptide library according to the protocols described in Examples 1, 2 and 3.

10

Phage clones displaying unique peptide sequences are isolated. These clones bind specifically to an above referenced diseased organ, but not to the healthy corresponding organ.

15

The amino acid sequences of these unique displayed peptides are determined by sequencing the corresponding DNA of the phage. The corresponding peptides are synthesized as described in Example 4. These peptides (or peptide mimetics thereof) are used, alone or linked to anti-cancer agent, as anti-metastatic and anti-cancer agents.

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-67-

EXAMPLE 7: Biopanning of T lymphocytes from Chronic Lymphocytic Leukemia patients (T-CLL)

5 Lymphocytes derived from patients suffering from leukemia or lymphoma differ from normal, healthy derived lymphocytes as they express different or modified epitopes on their surface.

Antibody library source

10 A human antibody synthetic library (Nissim-N1 library, EMBO J., 13:692, 1994) was provided by Dr. Ahuva Nissim. The diverse repertoire of rearranged V_H genes was built in-vitro by PCR from a bank of human V_H gene segments derived from 49 individual germ lines and random nucleotide sequence encoding
15 CDR3 lengths of 4-12 residues. The amplified rearranged V_H fragments were cloned with a single unmutated V3 segment derived from the germline IGLV3S1 (isolated from an antibody binding to BSA), as a single chain F_v fragment for phage display. This procedure created a single pot phagemid
20 library of more than 10^8 different clones and is displayed on the N-terminus of the pIII of the M13 bacteriophage.

Panning strategy and results

25 Lymphocytes isolated from the peripheral blood of T-CLL patient were biopanned with the single pot phagemid antibody library as follows: For the first round of selection, approximately 10^7 T-CLL cells were washed, blocked with PBS containing 4% skim milk and M13 and panned with 10^{12} phagemids from the N1 library, in the presence of wild-type
30 M13 (10^{13}). After incubation at 4°C for 1hr with slow agitation, cells were washed four times with PBS/milk, and the bound phagemids were eluted from the cells at room temperature for 5 min with 0.1M glycine pH-2.2. After neutralization, cells were spun, discarded, and the
35 supernatant containing the eluted phagemids was collected and was designated E1 stock.

The subsequent rounds were performed with 2×10^6 T-CLL cells.

WO 98/39469

PCT/US98/04188

-68-

a) The phagemids in E1 output were amplified by infecting E. coli bacteria (TG-1 strain) and plating them on ampicillin agar plates. Phagemids were rescued by superinfecting exponentially growing amp^r bacteria with helper phage (VSC-M13, Stratagene). This amplified stock-E1 was used for three additional rounds of panning, except that no amplification step was involved after each round of panning. After the last round of panning, the output phagemid was amplified and designated E4/1. Phagemids binding at high rate to T-CLL cells were characterized by DNA sequencing.

Twenty phagemid clones picked randomly from the E4/1 output were grown individually and a phagemid stock was prepared. These phagemid clones were mixed at a 1:1 molar ratio and used in three sequential rounds of panning on T-CLL cells and on normal blood lymphocytes (NBL).

All the phagemid clones tested which are derived from the second and from the third output were identical and code for a 10-mer CDR3 insert, whereas all 14 clones derived from second panning of the NBL were different from each other, and also not similar to that obtained in the T-CLL outputs. The repeating clone obtained in this procedure was designated JTCLL-IC#2. DNA sequencing of this clone was performed.

Amino acid sequence displayed in the CDR3 of clone JTCLL-IC#2:

Arg	Ser	Lys	Tyr	Arg	Pro	Asn	Met	Thr	Asn
1	2	3	4	5	6	7	8	9	10

b) The amplified E1 phagemid stock was used for two sequential rounds of panning as above, except that after washing, the bound phagemids were eluted for 15 min at room temperature with either PBS containing 10mM EDTA (E1ED2 stock) or with PBS containing 10mM ATP (E1AT2 stock).

DNA of phagemid clones derived from the ATP elution-E1AT2

WO 98/39469

PCT/US98/04188

-69-

stock (15 clones) and from the EDTA elution-E1ED2 stock (22 clones) were sequenced. Clones JTCLL-EDAT#10 and JTCLL-EDAT#22 appeared in both stocks E1AT2 and E1ED2 in 4/ 37 and 5/37 of the tested clones, respectively. The CDR3 encoded by these repeating clones is as follows:

Amino acid sequence displayed in the CDR3 of clone JTCLL-EDAT#10:

Leu Asn Pro Lys Val Lys His Met
10 1 2 3 4 5 6 7 8

Amino acid sequence displayed in the CDR3 of clone JTCLL-EDAT#22:

Leu Arg Gly Gly Asn Ala Met
15 1 2 3 4 5 6 7

c) Prior to amplification, the E1 output was used for two additional rounds of panning. The output phagemids were then amplified as above to give E3 stock. This E3 amplified stock (derived from only 300 different colonies) was used for three additional sequential rounds of panning and then amplified (E3CIII stock). In parallel, the last procedure was carried out also with NBL. After infection, the output from this procedure was designated E3NIII.

25 Clones derived from E3CIII and E3NIII outputs were sequenced: (i) all 23 clones tested from the E3NIII output were identical and designated JTCLL-T#29 (ii) in the E3CIII output 9/21 were identical to JTCLL-T#29 and 5/21 clones were designated JTCLL-E3C#2.

30

WO 98/39469

PCT/US98/04188

-70-

Amino acid sequence displayed in the CDR3 of clone JTCLL-T#29:

5 Val Ser Asp Arg Arg Gln Asn Val
 1 2 3 4 5 6 7 8

Amino acid sequence displayed in the CDR3 of clone JTCLL-E3C#2:

10 Ser Lys Ser Pro
 1 2 3 4

15 All peptides corresponding to the displayed peptides are synthesized as described in Example 4. These peptides (or peptide mimetics thereof), alone or linked to anti-cancer agent or to a radioisotope, are used as anti-cancer agents or for tumor diagnostics.

-71-

EXAMPLE 8: Biopanning of human lymphocytes for predetermined targets (CD44 variants) with a phage peptide library

5 Background

Numerous adhesion molecules are expressed by tumor cells and a variety of adhesive interactions are mediated by them. These adhesion molecules act as both positive and negative
10 modulators of invasive and metastatic processes. One of these adhesion proteins is CD44, a transmembrane glycoprotein functioning as the principal receptor for glycosaminoglycan hyaluronate (HA) and playing an important role in cell-extracellular matrix interactions.

15 CD44 is a ubiquitous multistructural and multifunctional cell surface adhesion molecule involved in cell-cell and cell-matrix interactions. Twenty exons are involved in the genomic organization of this molecule. The first five and
20 the last five exons are constant, whereas the 10 exons located between this region are subjected to alternative splicing, resulting in the generation of a variable region. Differential utilization of the 10 variable region exons generate multiple isoforms (at least 20 are known) of
25 different molecular sizes (85-230 kDa). The smallest CD44 molecule (85-95 kDa), which lacks the entire variable region, is standard CD44 (CD44s). As it is expressed mainly on cells of lymphohematopoietic origin, CD44s is also known as hematopoietic CD44 (CD44H). CD44s is a single chain
30 molecule composed of a distal extracellular domain (containing the ligand binding sites), a membrane proximal region, a transmembrane spanning domain, and a cytoplasmic tail. The molecular sequence (with the exception of the membrane proximal region) displays high interspecies
35 homology. After immunological activation, T lymphocytes and other leukocytes transiently upregulate CD44 isoforms expressing variant exons (designated CD44v). A CD44 isoform containing the last 3 exon products of the variable region

-72-

(CD44V8-10, also known as epithelial CD44 or CD44E), is preferentially expressed on epithelial cells. The longest CD44 isoform expressing in tandem eight exons of the variable region (CD44V3-10) was detected in keratinocytes.

5

Hyaluronic acid (HA), an important component of the extracellular matrix (ECM), is the principal, but by no means only ligand of CD44. Other CD44 ligands include the ECM components collagen, fibronectin, laminin, and chondroitin sulfate. Mucosal addressin, serglycin, osteopontin, and the class 2 invariant chain are additional, ECM-unrelated, ligands of the molecule. However, the polymorphic nature of CD44 suggests that this list is far from complete. Such hypothetical, novel CD44 ligands could be involved in tumor cell-tumor cell, tumor cell-normal cell, or tumor cell-matrix interactions, and their identification may allow the design of competitive or antagonistic reagents with potential antimetastatic effect.

In tumor cells, an overproduction of large CD44v proteins by enhanced transcription of alternatively spliced variant CD44v mRNA has been observed. Increased expression of CD44v proteins correlates with advanced stages of human breast, uterine, cervical and colon cancer, and has been found in human carcinoma lines from lung, breast and colon. In breast and colon cancer, expression of epitopes encoded by exons V5 or V6 on primary tumors is an independent prognostic factor for poor patient survival (Dall et al. (1995), Int. J. Cancer 66: 471-477; Rodriguez et al. (1995), Int. J. Cancer 64: 347-354; Liu (1994), Cancer Letters 76: 63-69).

Cells from the human Namalwa cell line (a Burkitt lymphoma derived lymphocyte) do not produce CD44 and are not metastatic. Upon transfection with CD44v (exon v6) cDNA, they gain the potential to colonize the lungs in an experimental metastasis protocol. Monoclonal antibodies specific for the v6 epitope, injected intravenously, prevent the outgrowth of CD44v6 transfectants in the draining lymph

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-73-

nodes and the formation of metastases in the lung and elsewhere (Herrlich et al. (1993), Immunology Today 14(8): 395-399).

5 The hyaluronate receptor CD44 has been shown to be involved
in lymphocyte homing during normal leucocyte circulation and
during leucocyte extravasation into sites of tissue
inflammation (Welsh et al. (1995) J. Cellular Physiol. 164:
605-612). Activated CD4⁺ or CD8 lymphocytes as well as
10 non-activated lymphocytes express CD44s (standard) while
CD44v (especially v6) is expressed only by memory T-
lymphocytes CD4⁺ and CD8⁺ (Tiberghien et al. (1994),
Immunology 83(4): 552-561). When activated, memory T-
lymphocytes express large quantities of CD44v protein on
15 their surface and produce strikingly higher amounts of
interferon-gamma (IFN- γ) and interleukin-4 (IL-4). Increased
concentrations of IFN- γ and IL-4 may contribute to the
formation of various autoimmune syndromes since these
cytokines may effect the production of certain classes of
20 antibodies (autoimmune antibodies) by augmenting MHC class
II expression (Budd et al. (1991), Eur. J. Immunol. 21(4):
1081-1084).

The role of the CD44 receptor in autoimmune disease and
25 inflammation has been studied in several animal models using
monoclonal antibodies (Mabs) which have been directed
against CD44s or CD44v (especially the v6 variant). In
collagen type II-induced arthritis in mice, injection of a
monoclonal anti-CD44 antibody suppressed the autoimmune
30 disease (Verdrengh et al. (1995), Scan. J. Immunol. 42(3):
353-358).

Similar results were obtained in rats with experimentally
induced-glomerulonephritis, immunized with bovine glomerular
35 basement membrane proteins (Nishikawa et al. (1993), Lab.
Invest. 68(2): 146-153) as well as in mice which develop a
syndrome similar to systemic lupus erythematosus in man.
This strain of mice is characterized by the progressive

-74-

accumulation of CD4⁺CD8⁺ T-cells which express increased levels of CD44v on their surface. These T-cells exhibit a high level of spontaneous cytolytic activity and contain high levels of serine esterase as compared with CD4⁺CD8⁺ T-cells. Treatment of these mice with anti-CD44v Mab augments the cytolytic activity of the CD4⁺CD8⁺ T-cells of the mice and causes suppression of their systemic lupus erythematosus like syndrome (Wang et al. (1993), Int. Immunol. 5(4): 361-369).

10

Methods

Antibody library source

A human antibody synthetic library as described in Example 7 was used.

15

Panning strategy and results

(A) Namalwa cells transfected with CD44V3-10 (Nam.+CD44V3-10) were panned with the single pot phagemid antibody library as follows: for the first round of selection, approximately 5×10^6 Nam.+CD44V3-10 cells were washed, blocked with PBS containing 4% skim milk and M13 and panned with 10^{12} phagemids from N1 library. After incubation at 4°C for 3h with slow agitation, cells were washed four times with PBS and the bound phages were eluted from the cells at room temperature for 10 min with 0.1M glycine pH-2.2. After neutralization, cells were spun, discarded, and the supernatant containing the eluted phages was collected and amplified by infecting E.coli bacteria (TG-1 strain) and plating them on ampicillin agar plates. Phagemids were rescued by superinfecting exponentially growing amp^r bacteria with helper phage (VSC-M13, Stratagene). This amplified stock was used for four additional rounds of panning on Namalwa cells as negatives and Nam.+CD44V3-10, with one amplification step after the second panning and without amplification step after the further rounds.

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4 peptides were isolated by this experiment:

WO 98/39469

PCT/US98/04188

-75-

Amino acid sequence displayed in the CDR3 of clone IG-NC-1:

Gly Thr Leu Asn Gln Cys Gly Arg Ile Asn
 1 2 3 4 5 6 7 8 9 10

5 Amino acid sequence displayed in the CDR3 of clone IG-NC-3:

Cys Ala Val Glu Ala Ala Gly Pro Val Arg Val Leu
 1 2 3 4 5 6 7 8 9 10 11 12

Amino acid sequence displayed in the CDR3 of clone IG-NC-14:

10 Ser Gly Ser Leu Gly Arg Ser Leu Glu
 1 2 3 4 5 6 7 8 9

Amino acid sequence displayed in the CDR3 of clone IG-NC-11:

15 Thr Gly Asp Glu
 1 2 3 4

(B) Namalwa/CHO-K1 cells which transfected with CD44V3-10 were panned with the single pot phagemid antibody library using the protocol of work described in (A) in the following approach:
 20

For the first round of selection approximately 5×10^6 Nam.+CD44V3-10 cells were washed, blocked with PBS containing 4% skim milk and M13 and panned with 10^{12} phagemids from N1 library. The bound phages were eluted from the cells and the supernatant containing the eluted phages was collected and divided into two steps: one including amplification step by infecting E.coli bacteria, and second step was done without amplification. For the second round of selection approximately 5×10^6 Namalwa/CHO-K1 cells were washed, blocked with PBS containing 4% skim milk and M13 and panned with phagemids eluted from the first selection round. The phagemids which did not bound to the Namalwa/CHO-K1 cells were incubated with Namalwa/CHO-K1 cells transfected with CD44V3-10 respectively. The second round of selection was repeated twice.
 25
 30
 35

6 peptides were isolated by this experiment:

WO 98/39469

PCT/US98/04188

-76-

Amino acid sequence displayed in the CDR3 of clone IG-NC-39:

Phe Lys Ala Ser Arg His Ser

1 2 3 4 5 6 7

- 5 Amino acid sequence displayed in the CDR3 of clone IG-NC-467:

Ile His Met Arg Ala

1 2 3 4 5

- 10 Amino acid sequence displayed in the CDR3 of clone IG-NC-471:

Lys Asn Ala Asn

1 2 3 4

- 15 Amino acid sequence displayed in the CDR3 of clone IG-NC-650:

Met Arg Ala Pro Val Ile

1 2 3 4 5 6

- 20 Amino acid sequence displayed in the CDR3 of clone IG-NC-651:

Gly Ile Lys Gly Leu Asp Glu

1 2 3 4 5 6 7

WO 98/39469

PCT/US98/04188

-77-

Amino acid sequence displayed in the CDR3 of clone IG-NC-652:

Cys Lys Trp Glu Lys Arg

5 1 2 3 4 5 6

10 All the peptides isolated are synthesized as described in Example 4. These peptides (or peptide mimetics thereof), alone or linked to an anti-cancer agent are used as anti-metastatic agents. In addition, these peptides are used for the treatment of autoimmune diseases, alone or linked to a suitable drug to form a drug-peptide conjugate.

-78-

EXAMPLE 9: Isolation of peptides which specifically bind to undetermined targets on the blood vessels of Kaposi sarcoma (KS) tumor-bearing nude mice in vivo.

5

Phage selection strategy:

10 For the induction of KS tumors we used a KS cell line which was isolated from a non-HIV-infected patient, and which was recently shown to carry several markers of HIV-infected KS cells (Herndier et al. (1994), Aids 8(5): 575-581). The KS cells (5×10^6) as a mixture in Matri-gel (1:1 v/v) were injected into nude mice. After approximately 12-14 days, when well defined localized tumors were visualized (about 15 1.2 cm in diameter), a mixture of the 15-mer library (described in Example 1) together with M13 bacteriophage (10^{12} : 10^{13} , respectively) was injected into the tail-vein. After 4 minutes, the animal vascular system was perfused with PBS for an additional 4 minutes and then sacrificed. 20 Tumors were excised, weighed, and kept on ice for all subsequent manipulations. Following homogenization and extensive washing, phage elution was carried out with starved bacteria. The eluted phage was titrated, amplified, and then subjected to additional rounds of 25 biopanning in KS tumor bearing mice.

Results of biopanning in vivo

30 The KS-tumor eluted phages from two independent experiments (designated MKS1 and MKS2), following three rounds of biopanning in vivo, were analyzed by DNA sequencing. The sequencing results indicated that almost 100% of the clones at round 3 in both experiments, were identical to clone TUV-R4B*#1 (Example 1), which was also selected on umbilical 35 vein and artery, ex vivo.

WO 98/39469

PCT/US98/04188

-79-

Organ distribution of selected phages:

5 Mixtures of selected phage and M13 bacteriophage (5×10^{10} and
5 5×10^{11} , respectively) were injected into the tail vein of KS-
tumor bearing mice, and after one round of panning, tumor
and brain tissue were excised, washed, and treated for phage
elution and titration as described above. The results of
three parallel experiments, with various phage mixtures, are
10 demonstrated in Figure 1.

These results demonstrate that the tumor-distribution of
clone TUV-R4B*#1 is 4-5 fold higher than the brain-
distribution. No such results were obtained with the two
other clones, which demonstrate similar low level of binding
15 to both tumor and brain tissues.

WO 98/39469

PCT/US98/04188

-80-

EXAMPLE 10: Isolation of peptides which specifically bind to undetermined targets on Kaposi sarcoma cells in culture.

5 Biopanning was conducted on a Kaposi sarcoma cell line (Herndier et al. (1994), Aids 8(5): 575-581) according to protocol EC-1 (section 1.5.5 in Example 1) with the following modifications: the selection was carried out with a mixture of 2×10^{10} phages of the 15-mer library (Example 1) and 5×10^{11} M13 wild-type phage. Phage selection (incubation) 10 was conducted at room-temperature for 60 minutes. Phage elution from the cells was carried out with starved bacteria only. Amplified phage was subjected to two additional cycles of biopanning. The DNA of 18 single phage clones from the eluate of the third cycle of biopanning was subjected to 15 DNA sequencing analysis.

10/28 clones were unique while 8/28 clones were identical and were designated TSKC-R3#3.

20 Amino acid sequence displayed by clone TSKC-R3#3:

Ala	Arg	Leu	Ser	Pro	Thr	Met	Val	His	Pro	Asn	Gly	Ala	Gln	Pro
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15

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-81-

EXAMPLE 11: Isolation of peptides that specifically bind to a predetermined target (fibronectin)

Biopanning was conducted on human plasmatic fibronectin (FN), (see eg. Akiyama and Yamada (1987), Adv. Enzymol 57: 1-57) essentially according to protocol EC-1 (section 1.5.5 in Example 1) with the following modifications: 35mm plates were coated overnight at 4°C with 25µg FN solution (in 5ml PBS). Plates were blocked with 5% Human Serum (HS) in PBS for 2 hours at room temperature, and washed 3 times with washing solution (5% HS-0.2% Tween in PBS). The phage selection was carried out with a mixture of 2×10^{10} TTU of the 15-mer library (Example 1) and 2×10^{11} PFU M13. Phage selection (incubation) was conducted at room-temperature for 60 minutes. Plates were washed 5 times with washing solution (5% HS-0.2% Tween in PBS). Phage elution was carried out in two steps for 10 minutes each at room temperature with:

Elution buffer 1: 0.2N glycine, pH2.2-0.5% Tween in PBS, followed by immediate neutralization of the acidic eluate with Trisma base.

Elution buffer 2: 0.05% EDTA in PBS.

Elutions 1 and 2 were mixed before bacterial infection and amplification.

The DNA of 16 single phage clones isolated from the third cycle of biopanning was analyzed by DNA sequencing.

8/16 clones were identical and were designated TFN-R3-#150.

4/16 clones were identical and were designated TFN-R3-#111.

4/4 clones were unique.

WO 98/39469

PCT/US98/04188

-82-

Amino acid sequence displayed by clone TFN-R3-#150:

Ala Leu Gly Gly Phe Arg Pro Phe Trp Ser Tyr Gly Gly Leu Ser
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

5

Amino acid sequence displayed by clone TFN-R3-#111:

Met Gly Ala Asp Asp Ala Pro His Tyr Trp His Pro Val Trp Thr
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

10

-83-

EXAMPLE 12: Isolation of peptides which specifically bind to undetermined targets on human Acute Myeloid Leukemia (AML) cells.

5 **Biopanning protocol**

Frozen AML lymphocytes, which were originally isolated by Ficol-density centrifugation from the peripheral blood cells of an Acute Myeloid Leukemic patient, were quickly thawed at 37°C water bath, and washed in washing medium (DMEM-10% Human serum (HS)). The viability estimated by trypan blue exclusion was 75-90%. The AML cells were resuspended in selection medium (Hanks-2 mM HEPES, pH 6.9 and 2% HS). Approximately, 5×10^{11} TTU (from the 15 or the 6-mer library, Example 1) were added to 5×10^6 cells in a final volume of 1.4ml medium in an Eppendorf tube, and the mixture was incubated at 4°C, for 60 minutes while rocking. The cells were then washed with DMEM medium, and the Eppendorf tube was changed after the second washing. Washing was repeated four times. Phage elution from the cells was carried out with Trypsin-EDTA (200µl, Gibco) at 37°C for 15 minutes, followed by the addition of HS (10% final concentration) for trypsin neutralization. Phage titrations in the input and the eluate (output), and phage amplification for additional rounds of biopanning, were carried out according to G. Smith (Virology 167: 156-165, 1988).

Biopanning on AML lymphocytes with the 15-mer library:

30 In rounds 2-4, the amplified output (eluted phage) was used as the input phage for the subsequent round of biopanning.

The DNA of 28 single clones was analyzed by DNA sequencing after the fifth round of selection.

35

17/28 clones were identical and were designated EAML.5.73.
11/28 clones were unique.

WO 98/39469

PCT/US98/04188

-84-

Amino acid sequence displayed by clone EAML.5.73.:

Cys Thr Arg Leu Gly Ala Ala Ala Gly Arg Cys Asp Val Gly Leu
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

5 The DNA of 9 clones was analyzed by DNA sequencing after the sixth round of selection.

2/9 clones were identical and were designated EAML.6.1.

Amino acid sequence displayed by clone EAML.6.1.:

10 Ser Asn Leu Asn Arg Phe Val Phe Ala Phe Trp Asp Gly Pro Ala
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Biopanning on AML lymphocytes with a 1:1 mixture of the 15 and 6-mer libraries

15

28 single colonies from round 6 were sequenced.

20/28 clones were identical and were designated EAML.6.108.

Amino acid sequence displayed by clone EAML. 6.108:

20 Arg Leu Phe Met Leu Gly
1 2 3 4 5 6

An additional experiment was carried out as above, in which 5 single colonies from round 6 were sequenced.

25 3/5 clones were identical and were designated EAML 6.2.

2/5 clones were identical and were designated EAML 6.4

Amino acid sequence displayed by clone EAML.6.2.:

30 Leu His Gly Phe Ala Ser His Lys Asp Gly Pro Leu Ile Pro Ala
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Amino acid sequence displayed by clone EAML.6.4.:

Leu Val Phe Val Lys Asn His Pro Leu Val Pro Phe Gly Ser Pro
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

35

The comparative binding of the AML-selected clones EAML.5.73 and EAML.6.108 to AML cells from three different AML patients was 10-100 times higher than the original 15-mer

WO 98/39469

PCT/US98/04188

-85-

and 6-mer libraries.

-86-

EXAMPLE 13: Isolation of peptides which specifically bind to undetermined targets on human chronic lymphoid leukemia (T-CLL) cells.

5 **Biopanning strategy:**

Biopanning on CLL3 cells was conducted with the 15-mer library (Example 1, 2×10^6 cells, 5×10^9 phage, 2×10^8 M13, 4°C, 60min, Hanks-Heppes). Biopanning followed by
10 amplification was carried out for several rounds.

Round 1: Elution of bound phage with acid-glycine followed by trypsin-EDTA. The two eluates were mixed, amplified and then used for the second round.

Round 2: Phage eluted with acid-glycine and then with
15 trypsin-EDTA. Each eluate was amplified and used separately for the next rounds of biopanning.

Round 3-5: When the amplified acid eluate from round 2 was used for biopanning, phage elution was carried with acid-glycine. When the amplified trypsin-EDTA eluate was used
20 for biopanning, bound phage was washed with acid-glycine, and elution was carried out with trypsin-EDTA.

Specific phage enrichment was monitored by DNA sequencing and by binding assays.

25

Three peptides were isolated by acid elutions:

Amino acid sequence displayed by clone TCELL.HA4.6:

Pro Ile Trp His Gly Asp Ser Gly Val Tyr Ser Ser Phe Phe Pro
30 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Amino acid sequence displayed by clone TCELL.HA5.5:

Arg Asn Thr Leu Pro His Phe Ser Phe Gly Pro Arg Leu Tyr Arg
35 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

WO 98/39469

PCT/US98/04188

-87-

Amino acid sequence displayed by clone TCLL.HA3.11:

Gly Leu Ser Asp Gly pro Tyr Tyr Ser Phe Ser Leu Phe Arg Phe
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

5

Two peptides were isolated by trypsin-EDTA elutions:

Amino acid sequence displayed by clone TCLL.HT3.2:

Gly Gly Ala Ala Gly Gly Tyr Leu Arg Val Phe Ala Gly Val Arg
10 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Amino acid sequence displayed by clone TCLL.HT4.13:

Gly Tyr Asp Cys Trp Asp Cys Pro Phe Ser Phe Arg Gly Ser Val
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

EXAMPLE 14: Isolation of peptides that specifically bind to
Thrombospondin

5

Background

Thrombospondin (TSP) is a glycoprotein which is stored in
platelets α -granules, and generally released in response to
10 thrombin stimulation. TSP functions as a cell adhesion
molecule as well as a modulator of cell movement and
proliferation. There is growing evidence that TSP functions
as a natural modulator of angiogenesis, leading to positive
or negative regulation of angiogenesis, and as a function of
15 context and TSP concentration. Moreover, TSP is
overexpressed by tumors, and more specifically, high levels
of TSP were observed in association with malignant
carcinomas of the breast. TSP molecules are also a transient
component of extracellular matrix in developing and
20 repairing tissues. This process is mediated by binding of
the amino-terminal heparin-binding domain of TSP to the
extracellular matrix and cell surface proteoglycans.

Biopanning strategy

25 Biopanning was conducted on the recombinant heparin-binding-
domain of human TSP-1 (18 kD) designated rTSP-HBD1 (Vogel et
al. (1994), J.Cell.Biochem., 53:75), which spans amino acids
1-174 of human TSP-1. Biopanning was carried out essentially
according to protocol EC-1 (Example 1) with the following
30 modifications: For round 1, 35mm plates were coated
overnight at 4°C with 25 μ g solution (in 5ml PBS) of rTSP-
HBD1. Plates were blocked with 2% skim-milk (SM) in PBS for
45 minutes at 37°C and 45 minutes at room temperature, and
washed 3 times with washing solution (PBS). Phage selection
35 was carried out with a mixture of approximately 2×10^{10} TTU of
the 15-mer library (Example 1) and 2×10^{11} PFU M13, or with
 10^{11} Nissim N-1 library (Example 7) in 2% SM-PBS. Incubation
was carried out at room temperature for 90 minutes. Plates

WO 98/39469

PCT/US98/04188

-89-

were washed 5 times with 0.2% Tween-PBS, followed by 5 washings with PBS. Phage elution was carried out for 10 min at room temperature with:

- 5 Elution buffer 1: 0.2N glycine (pH2.2)-0.2% Tween, followed by immediate neutralization with Trisma base.

Elution buffer 2: 5×10^9 starved K91 bacteria for 30 min at room temperature.

10

Elutions 1 and 2 were mixed before amplification. For the subsequent rounds, the protocol was as described above except that the plates were coated with $5 \mu\text{g}$ solution of rTSP-HBD1, and phage incubation was carried out for 45 minutes. Elutions 1 and 2 were amplified and used separately for rounds 3 and onwards.

15

DNA analysis of 17 phage clones (Nissim N-1 library) was carried out after round 3 (elution buffer 2):

20

3/17 clones were identical and were designated TTSP.R3N.B5.

Amino acid sequence displayed in the CDR3 of TTSP.R3N.B5:

Ser Lys Arg Ala Asn Gly Phe Arg Gly Val Ser

25

1	2	3	4	5	6	7	8	9	10	11
---	---	---	---	---	---	---	---	---	----	----

1/17 clones was designated TTSP.R3N.B3.

Amino acid sequence displayed in the CDR3 of TTSP.R3N.B3:

30

Ile Lys His Tyr Gly Arg Lys Arg Asn

1	2	3	4	5	6	7	8	9
---	---	---	---	---	---	---	---	---

1/17 clones was designated TTSP.R3N.B4.

35

Amino acid sequence displayed in the CDR3 of TTSP.R3N.B4:

Val Lys Lys Phe Lys Gly Gly Gln Arg Val

WO 98/39469

PCT/US98/04188

-90-

1 2 3 4 5 6 7 8 9 10

	clone	Frequency	Sequence
5	-----		-----
	TTSP.R3N.B5	3/17	S K R A N G F R G V S
	TTSP.R3N.B3	1/17	I K H Y G R K R N
10	TTSP.R3N.B4	1/17	V K K F K G G Q R V
	-----		-----

The bold and italics signs designate the consensus amino acids of the selected peptide ligands.

-91-

What is claimed is:

1. A non naturally occurring pharmaceutically active peptide.

5

2. A peptide of claim 1 comprising amino acids having the sequence:

Gly Arg Gln His Phe Phe Leu Ala Glu Gly Arg Ser Phe Tyr
Phe

10

3. A peptide according to claim 2 having the amino acid sequence:

Gly Arg Gln His Phe Phe Leu Ala Glu Gly Arg Ser Phe Tyr
Phe

15

4. A peptide of claim 1 comprising amino acids having the sequence:

Ser His Val Pro Pro Ile Phe Asn Asp Val Tyr Trp Ile Ala
Phe

20

5. A peptide according to claim 4 having the amino acid sequence:

Ser His Val Pro Pro Ile Phe Asn Asp Val Tyr Trp Ile Ala
Phe

25

6. A peptide of claim 1 comprising amino acids having the sequence:

Val Pro Pro Ile Phe Asn Asp Val Tyr Trp Ile Ala Phe

30

7. A peptide according to claim 6 having the amino acid sequence:

Val Pro Pro Ile Phe Asn Asp Val Tyr Trp Ile Ala Phe

35

8. A peptide of claim 1 comprising amino acids having the sequence:

His Thr Phe Phe Leu Pro Gly Cys Ala Gly His Cys Ile Asp
Ala

WO 98/39469

PCT/US98/04188

-92-

9. A peptide according to claim 8 having the amino acid sequence:
His Thr Phe Phe Leu Pro Gly Cys Ala Gly His Cys Ile
Asp Ala
- 5 10. A peptide of claim 1 comprising amino acids having the sequence:
Pro Ser Thr Thr Arg Asn Arg Thr Asp Ile Asn Lys Pro
Thr Gln
- 10 11. A peptide according to claim 10 having the amino acid sequence:
Pro Ser Thr Thr Arg Asn Arg Thr Asp Ile Asn Lys Pro
Thr Gln
- 15 12. A peptide of claim 1 comprising amino acids having the sequence:
Phe Tyr Ser His Ser Ala Asp Gly Ala Arg Pro Phe Pro
Leu Tyr
- 20 13. A peptide according to claim 12 having the sequence:
Phe Tyr Ser His Ser Ala Asp Gly Ala Arg Pro Phe Pro
Leu Tyr
- 25 14. A peptide of claim 1 comprising amino acids having the sequence:
Phe Tyr Ser His Ser Ala Asp Gly Ala Glu Ser Ser Pro
Arg Met
- 30 15. A peptide according to claim 14 having the sequence:
Phe Tyr Ser His Ser Ala Asp Gly Ala Glu Ser Ser Pro
Arg Met
- 35 16. A peptide of claim 1 comprising amino acids having the sequence:
Phe Tyr Ser His Ser Ala Asp Gly Ala Pro Arg Arg Asp

WO 98/39469

PCT/US98/04188

-93-

Leu Leu

17. A peptide according to claim 16 having the sequence:

5 Phe Tyr Ser His Ser Ala Asp Gly Ala Pro Arg Arg Asp
Leu Leu

18. A peptide of claim 1 comprising amino acids having the sequence:

10 Phe Tyr Ser His Ser Ala Asp Gly Ala

19. A peptide according to claim 18 having the sequence:

15 Phe Tyr Ser His Ser Ala Asp Gly Ala

20. A peptide of claim 1 comprising amino acids having the sequence:

20 Phe Tyr Ser His Ser Ala Asp Gly Ala XXX XXX XXX Pro
XXX XXX, wherein XXX is any amino acid.

21. A peptide according to claim 20 having the sequence:

25 Phe Tyr Ser His Ser Ala Asp Gly Ala XXX XXX XXX Pro
XXX XXX, wherein XXX is any amino acid.

22. A peptide of claim 1 comprising amino acids having the sequence:

30 Phe Tyr Ser His Ser Ala Asp Gly Ala XXX XXX XXX XXX
Leu XXX, wherein XXX is any amino acid.

23. A peptide according to claim 22 having the sequence:

35 Phe Tyr Ser His Ser Ala Asp Gly Ala XXX XXX XXX XXX
Leu XXX, wherein XXX is any amino acid.

24. A peptide of claim 1 comprising amino acids having the sequence:

Gly Cys Gly Gly Phe His Pro Gly Ser Asn Cys Leu Val

WO 98/39469

PCT/US98/04188

-94-

Val Ser

25. A peptide according to claim 24 having the amino acid sequence:
5 Gly Cys Gly Gly Phe His Pro Gly Ser Asn Cys Leu Val Val Ser
26. A peptide of claim 1 comprising amino acids having the sequence:
10 Arg Asn Val Pro Pro Ile Phe Asn Asp Val Tyr Trp Ile Ala Phe
27. A peptide according to claim 26 having the sequence:
15 Arg Asn Val Pro Pro Ile Phe Asn Asp Val Tyr Trp Ile Ala Phe
28. A peptide of claim 1 comprising amino acids having the sequence: Phe Leu Pro Asn Gly Phe.
20
29. A peptide according to claim 28 having the amino acid sequence: Phe Leu Pro Asn Gly Phe.
30. A peptide of claim 1 comprising amino acids having the sequence: Thr His Asp Thr His Leu.
25
31. A peptide according to claim 30 having the amino acid sequence: Thr His Asp Thr His Leu.
- 30 32. A peptide of claim 1 comprising amino acids having the sequence: Thr His Glu Thr Gln Arg.
33. A peptide according to claim 32 having the amino acid sequence: Thr His Glu Thr Gln Arg.
35
34. A peptide of claim 1 comprising amino acids having sequence: Asp Ile Ala Lys Arg Tyr.

WO 98/39469

PCT/US98/04188

-95-

35. A peptide according to claim 34 having the amino acid sequence: Asp Ile Ala Lys Arg Tyr.
- 5 36. A peptide of claim 1 comprising amino acids having the sequence:
Asp Val Ser Pro Val Trp Ala Ala Phe Ala Ser Gly Ala Ser Phe
- 10 37. A peptide according to claim 36 having the amino acid sequence:
Asp Val Ser Pro Val Trp Ala Ala Phe Ala Ser Gly Ala Ser Phe
- 15 38. A peptide of claim 1 comprising amino acids having the sequence:
Ser Asp Cys Leu His Ser Val Arg Gly Phe Asn Cys Leu Lys Arg
- 20 39. A peptide according to claim 38 having the amino acid sequence:
Ser Asp Cys Leu His Ser Val Arg Gly Phe Asn Cys Leu Lys Arg
- 25 40. A peptide of claim 1 comprising amino acids having the sequence:
Ser Leu Cys Phe Tyr Leu Phe Val Met Ser Ala Pro Asp Ala Pro
- 30 41. A peptide according to claim 40 having the amino acid sequence:
Ser Leu Cys Phe Tyr Leu Phe Val Met Ser Ala Pro Asp Ala Pro

WO 98/39469

PCT/US98/04188

-96-

42. A peptide of claim 1 comprising amino acids having the sequence:
Gly Leu Gly Gly Leu Ser Phe Gly His Ser Asp Asn Pro
Pro Ser
- 5
43. A peptide according to claim 42 having the amino acid sequence:
Gly Leu Gly Gly Leu Ser Phe Gly His Ser Asp Asn Pro
Pro Ser
- 10
44. A peptide of claim 1 comprising amino acids having the sequence:
Gly Pro Gly Trp Val Gly Trp Phe Val Ser Leu Phe Tyr
Ala Ser
- 15
45. A peptide according to claim 44 having the amino acid sequence:
Gly Pro Gly Trp Val Gly Trp Phe Val Ser Leu Phe Tyr
Ala Ser
- 20
46. A peptide of claim 1 comprising amino acids having the sequence:
Trp Ser Leu Gly Ser Ser Trp Val Tyr Lys Phe Phe Tyr
Ser Ser
- 25
47. A peptide according to claim 46 having the sequence:
Trp Ser Leu Gly Ser Ser Trp Val Tyr Lys Phe Phe Tyr
Ser Ser
- 30
48. A peptide of claim 1 comprising amino acids having the sequence:
Ser Leu Trp Gly Ala Ser Ser Cys Gly Val Ala Phe Phe
Glu Ser
- 35
49. A peptide according to claim 48 having the sequence:
Ser Leu Trp Gly Ala Ser Ser Cys Gly Val Ala Phe Phe

-97-

Glu Ser

50. A peptide of claim 1 comprising amino acids having the sequence:

5 Gly Pos Pos Pos/Ar Ar Ar Leu Ala Glu Gly Arg Ser Ar
Ar Ar

wherein Pos is a positively charged amino acid and
Ar is an aromatic amino acid.

10

51. A peptide according to claim 50 having the amino acid sequence:

Gly Pos Pos Pos/Ar Ar Ar Leu Ala Glu Gly Arg Ser Ar
Ar Ar

15

wherein Pos is a positively charged amino acid and
Ar is an aromatic amino acid.

52. A peptide of claim 1 comprising amino acids having the sequence Arg Ser Lys Tyr Arg Pro Asn Met Thr Asn

20

53. A peptide according to claim 52 having the amino acid sequence Arg Ser Lys Tyr Arg Pro Asn Met Thr Asn

25

54. A peptide of claim 1 comprising amino acids having the sequence Leu Asn Pro Lys Val Lys His Met

30

55. A peptide according to claim 54 having the amino acid sequence Leu Asn Pro Lys Val Lys His Met

56. A peptide of claim 1 comprising amino acids having the sequence Leu Arg Gly Gly Asn Ala Met

35

57. A peptide according to claim 56 having the amino acid sequence Leu Arg Gly Gly Asn Ala Met

WO 98/39469

PCT/US98/04188

-98-

58. A peptide of claim 1 comprising amino acids having the sequence Val Ser Asp Arg Arg Gln Asn Val
- 5 59. A peptide according to claim 58 having the amino acid sequence Val Ser Asp Arg Arg Gln Asn Val
60. A peptide of claim 1 comprising amino acids having the sequence Ser Lys Ser Pro
- 10 61. A peptide according to claim 60 having the amino acid sequence Ser Lys Ser Pro
62. A peptide of claim 1 comprising amino acids having the sequence Gly Thr Leu Asn Gln Cys Gly Arg Ile Asn
- 15 63. A peptide according to claim 62 having amino acid sequence Gly Thr Leu Asn Gln Cys Gly Arg Ile Asn
- 20 64. A peptide of claim 1 comprising amino acids having the sequence Cys Ala Val Glu Ala Ala Gly Pro Val Arg Val Leu
- 25 65. A peptide according to claim 64 having amino acid sequence Cys Ala Val Glu Ala Ala Gly Pro Val Arg Val Leu
66. A peptide of claim 1 comprising amino acids having the sequence Ser Gly Ser Leu Gly Arg Ser Leu Glu
- 30 67. A peptide according to claim 66 having amino acid sequence Ser Gly Ser Leu Gly Arg Ser Leu Glu
68. A peptide of claim 1 comprising amino acids having the sequence Thr Gly Asp Glu
- 35 69. A peptide according to claim 68 having amino acid sequence Thr Gly Asp Glu
-

WO 98/39469

PCT/US98/04188

-99-

70. A peptide of claim 1 comprising amino acids having the sequence Phe Lys Ala Ser Arg His Ser
- 5 71. A peptide according to claim 70 having amino acid sequence Phe Lys Ala Ser Arg His Ser
72. A peptide of claim 1 comprising amino acids having the sequence Ile His Met Arg Ala
- 10 73. A peptide according to claim 72 having amino acid sequence Ile His Met Arg Ala
74. A peptide of claim 1 comprising amino acids having the sequence Lys Asn Ala Asn
- 15 75. A peptide according to claim 74 having amino acid sequence Lys Asn Ala Asn
76. A peptide of claim 1 comprising amino acids having the sequence Met Arg Ala Pro Val Ile
- 20 77. A peptide according to claim 76 having amino acid sequence Met Arg Ala Pro Val Ile
- 25 78. A peptide of claim 1 comprising amino acids having the sequence Gly Ile Lys Gly Leu Asp Glu
79. A peptide according to claim 78 having amino acid sequence Gly Ile Lys Gly Leu Asp Glu
- 30 80. A peptide of claim 1 comprising amino acids having the sequence Cys Lys Trp Glu Lys Arg
81. A peptide according to claim 80 having amino acid sequence Cys Lys Trp Glu Lys Arg
- 35 82. A peptide of claim 1 comprising amino acids having the sequence Ala Arg Leu Ser Pro Thr Met Val His

WO 98/39469

PCT/US98/04188

-100-

Pro Asn Gly Ala Gln Pro

- 5 83. A peptide according to claim 82 having amino acid sequence Ala Arg Leu Ser Pro Thr Met Val His Pro Asn Gly Ala Gln Pro
- 10 84. A peptide of claim 1 comprising amino acids having the sequence Ala Leu Gly Gly Phe Arg Pro Phe Trp Ser Tyr Gly Gly Leu Ser
- 15 85. A peptide according to claim 84 having amino acid sequence Ala Leu Gly Gly Phe Arg Pro Phe Trp Ser Tyr Gly Gly Leu Ser
- 20 86. A peptide of claim 1 comprising amino acids having the sequence Met Gly Ala Asp Asp Ala Pro His Tyr Trp His Pro Val Trp Thr
- 25 87. A peptide according to claim 86 having amino acid sequence Met Gly Ala Asp Asp Ala Pro His Tyr Trp His Pro Val Trp Thr
- 30 88. A peptide of claim 1 comprising amino acids having the sequence Cys Thr Arg Leu Gly Ala Ala Ala Gly Arg Cys Asp Val Gly Leu
- 35 89. A peptide according to claim 88 having amino acid sequence Cys Thr Arg Leu Gly Ala Ala Ala Gly Arg Cys Asp Val Gly Leu
90. A peptide of claim 1 comprising amino acids having the sequence Arg Leu Phe Met Leu Gly
91. A peptide according to claim 90 having amino acid sequence Arg Leu Phe Met Leu Gly
92. A peptide of claim 1 comprising amino acids having the sequence Pro Ile Trp His Gly Asp Ser Gly Val

-101-

Tyr Ser Ser Phe Phe Pro

- 5 93. A peptide according to claim 92 having amino acid sequence Pro Ile Trp His Gly Asp Ser Gly Val Tyr Ser Ser Phe Phe Pro
- 10 94. A peptide of claim 1 comprising amino acids having the sequence Arg Asn Thr Leu Pro His Phe Ser Phe Gly Pro Arg Leu Tyr Arg
- 15 95. A peptide according to claim 94 having amino acid sequence Arg Asn Thr Leu Pro His Phe Ser Phe Gly Pro Arg Leu Tyr Arg
- 20 96. A peptide of claim 1 comprising amino acids having the sequence Gly Leu Ser Asp Gly pro Tyr Tyr Ser Phe Ser Leu Phe Arg Phe
- 25 97. A peptide according to claim 96 having amino acid sequence Gly Leu Ser Asp Gly pro Tyr Tyr Ser Phe Ser Leu Phe Arg Phe
- 30 98. A peptide of claim 1 comprising amino acids having the sequence Gly Gly Ala Ala Gly Gly Tyr Leu Arg Val Phe Ala Gly Val Arg
- 35 99. A peptide according to claim 98 having amino acid sequence Gly Gly Ala Ala Gly Gly Tyr Leu Arg Val Phe Ala Gly Val Arg
100. A peptide of claim 1 comprising amino acids having the sequence Gly Tyr Asp Cys Trp Asp Cys Pro Phe Ser Phe Arg Gly Ser Val
101. A peptide according to claim 100 having amino acid sequence Gly Tyr Asp Cys Trp Asp Cys Pro Phe Ser Phe Arg Gly Ser Val

WO 98/39469

PCT/US98/04188

-102-

102. A peptide of claim 1 comprising amino acids having the sequence Ser Asn Leu Asn Arg Phe Val Phe Ala Phe Trp Asp Gly Pro Ala
- 5 103. A peptide according to claim 102 having amino acid sequence Ser Asn Leu Asn Arg Phe Val Phe Ala Phe Trp Asp Gly Pro Ala
- 10 104. A peptide of claim 1 comprising amino acids having the sequence Leu His Gly Phe Ala Ser His Lys Asp Gly Pro Leu Ile Pro Ala
- 15 105. A peptide according to claim 104 having amino acid sequence Leu His Gly Phe Ala Ser His Lys Asp Gly Pro Leu Ile Pro Ala
- 20 106. A peptide of claim 1 comprising amino acids having the sequence Leu Val Phe Val Lys Asn His Pro Leu Val Pro Phe Gly Ser Pro
- 25 107. A peptide according to claim 106 having amino acid sequence Leu Val Phe Val Lys Asn His Pro Leu Val Pro Phe Gly Ser Pro
- 30 108. A peptide of claim 1 comprising amino acids having the sequence Ser Lys Arg Ala Asn Gly Phe Arg Gly Val Ser
- 35 109. A peptide according to claim 108 having amino acid sequence Ser Lys Arg Ala Asn Gly Phe Arg Gly Val Ser
110. A peptide of claim 1 comprising amino acids having the sequence Ile Lys His Tyr Gly Arg Lys Arg Asn
111. A peptide according to claim 110 having amino acid sequence Ile Lys His Tyr Gly Arg Lys Arg Asn

WO 98/39469

PCT/US98/04188

-103-

112. A peptide of claim 1 comprising amino acids having the sequence Val Lys Lys Phe Lys Gly Gly Gln Arg Val
- 5 113. A peptide according to claim 110 having amino acid sequence Val Lys Lys Phe Lys Gly Gly Gln Arg Val
- 10 114. A composition comprising a peptide according to any of claims 2 to 113 and a pharmaceutical agent linked thereto.
- 15 115. A composition according to claim 114 wherein the pharmaceutical agent is a polypeptide and is linked to the peptide by a peptide linkage.
- 20 116. A composition according to claim 114 wherein the pharmaceutical agent is a toxin, an anti-cancer agent, an anti-angiogenic compound, a cardiovascular agent, an agent used in a neurological disorder, a liver disease agent, a kidney disease agent or a radioisotope.
- 25 117. A composition according to claim 114 wherein the pharmaceutical agent is a recombinant protein.
- 30 118. A composition comprising a peptide according to any of claims 2 to 113 and a pharmaceutically acceptable carrier.
- 35 119. A composition according to claim 114 which additionally comprises a pharmaceutically acceptable carrier.
120. A chimeric polypeptide comprising a first peptide and a second peptide wherein the first peptide is a peptide of any of claims 2-113.
121. A polypeptide according to claim 120 wherein the

PCT/US 98 / 04 188
IPEA/US 03 MAR 1999

-104-

second peptide is a toxin, an anti-cancer agent, an anti-angiogenic compound, a cardiovascular agent, an agent used in a neurological disorder, a liver disease agent or a kidney disease agent.

5

122. A polypeptide according to claim 120 wherein the second peptide is a recombinant protein.

10

123. A method for the identification of a peptide which comprises:

a. incubating a phage display peptide library with an isolated organ;

15

b. washing the isolated organ to remove unbound phages;

c. eluting bound phage from the isolated organ;

20

d. amplifying the resulting bound phage; and

e. determining the displayed peptide sequence of the bound phage so as to identify the peptide.

25

124. A method of synthesizing a peptide of any of claims 2-113 which comprises joining the amino acids of the peptide in the proper order.

30

125. A method of producing a peptide which comprises:

a. identifying the peptide by the method of claim 123; and

35

b. synthesizing the peptide by joining the amino acids of the peptide in the proper order.

126. A method according to claim 123 wherein the isolated organ is a perfused organ.

WO 98/39469

PCT/US98/04188

-105-

127. A method according to claim 123 wherein the isolated organ is an artery, a vein, placenta, tumor tissue, kidney, heart, liver, or central nervous system.
- 5 128. A method according to claim 127 wherein the artery is umbilical cord artery, a radial artery, a coronary artery or a mammary artery.
- 10 129. A method according to claim 127 wherein the artery is a damaged artery.
130. A method according to claim 129 wherein the damaged artery is a damaged coronary artery.
- 15 131. A method according to claim 127 wherein the vein is umbilical cord vein, safenal vein or femoral vein.
132. A method according to claim 123 wherein the phage display peptide library is a 15-mer library.
- 20 133. A method according to claim 123 wherein the phage display peptide library is a 6-mer library.
- 25 134. A method according to claim 123 wherein the elution medium is a compound selected from acid, urea, Octyl, trypsin or tween.
- 30 135. An imaging agent which comprises a peptide of any of claims 2-113 labeled with an imageable marker.
136. A composition comprising an effective imaging amount of the imaging agent of claim 135 and a physiologically acceptable carrier.
- 35 137. A composition comprising an effective imaging amount of the imaging agent of claim 135, a pharmaceutical agent linked thereto and a

WO 98/39469

PCT/US98/04188

-106-

physiologically acceptable carrier.

- 5 138. An agent according to claim 135 wherein the marker is a radioactive isotope, an element which is opaque to X-rays or a paramagnetic ion.
139. An agent of claim 138 wherein the marker is a radioactive isotope.
- 10 140. An agent of claim 139 wherein the radioactive isotope is indium-111, technetium-99, iodine-123, iodine-125, iodine-131, krypton-81m, xenon-33 or gallium-67.
- 15 141. A method for imaging an organ which comprises:
- (i) contacting the organ to be imaged with an imaging agent according to claim 135 under conditions such that the imaging agent binds to the organ ;
- 20 (ii) imaging bound imaging agent; and
- (iii) thereby imaging the organ.
- 25 142. A method according to claim 141 wherein the organ is an artery, a vein, placenta, tumor tissue, kidney, heart or liver.
- 30 143. A method according to claim 142 wherein the artery is umbilical cord artery, a radial artery, a coronary artery or a mammary artery.
144. A method according to claim 142 wherein the artery is a damaged artery.
- 35 145. A method according to claim 144 wherein the damaged artery is a damaged coronary artery.

WO 98/39469

PCT/US98/04188

-107-

146. A method according to claim 142 wherein the vein is umbilical cord vein, safenal vein or femoral vein.
- 5 147. A composition according to claim 137 wherein the pharmaceutical agent is a polypeptide and is linked to the imaging agent by a peptide linkage.
- 10 148. A composition according to claim 137 wherein the pharmaceutical agent is a toxin, an anti-cancer agent, an anti-angiogenic compound, a cardiovascular agent, an agent used in a neurological disorder, a liver disease agent or a kidney disease agent.
- 15 149. A composition according to claim 137 wherein the pharmaceutical agent is a recombinant protein.
- 20 150. A method of treating an organ *in vivo* which comprises:
- (i) contacting the organ to be treated with a composition according to claim 137 under conditions such that the composition binds to the organ; and
- 25 (ii) thereby treating the organ.
- 30 151. A method according to claim 150 wherein the organ is an artery, a vein, placenta, tumor tissue, kidney, heart, liver, or central nervous system.
152. A method according to claim 151 wherein the artery is umbilical cord artery, a radial artery, a coronary artery or a mammary artery.
- 35 153. A method according to claim 151 wherein the artery is a damaged artery.
154. A method according to claim 153 wherein the damaged

WO 98/39469

PCT/US98/04188

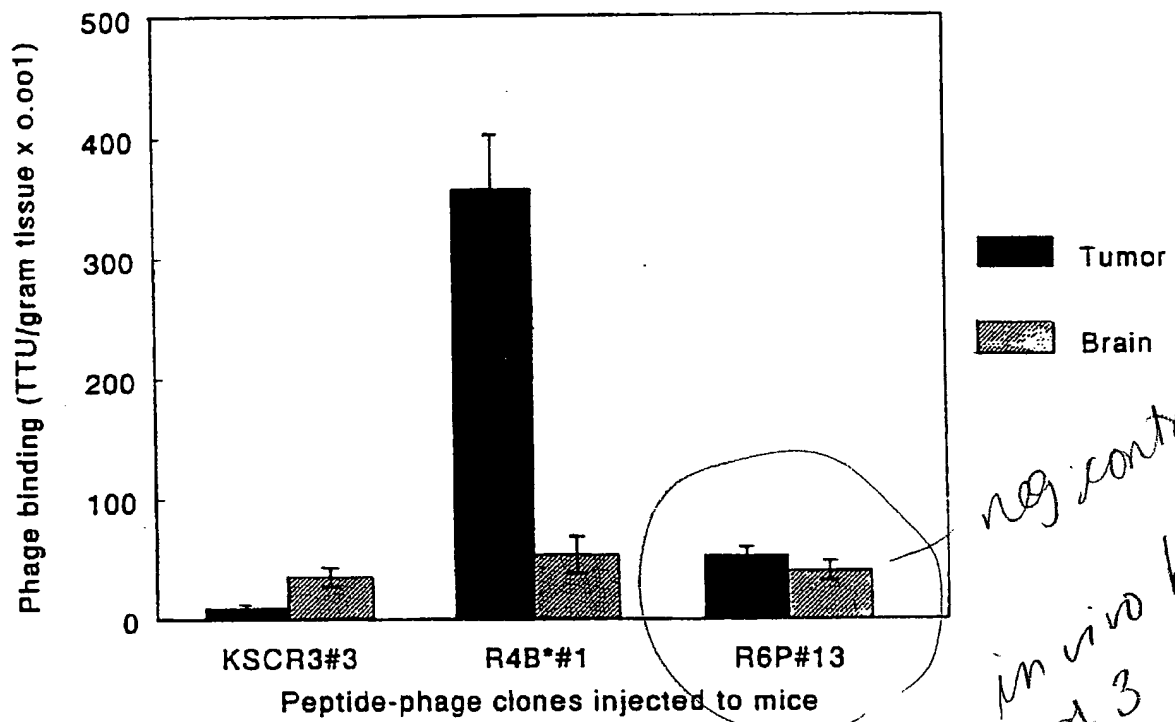
-108-

artery is a damaged coronary artery.

155. A method according to claim 151 wherein the vein is umbilical cord vein, safenal vein or femoral vein.

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FIGURE 1



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(54) ADMINISTRATION DE COMPOSES THERAPEUTIQUES AU CERVEAU ET A D'AUTRES TISSUS

(54) DELIVERY OF THERAPEUTIC COMPOUNDS TO THE BRAIN AND OTHER TISSUES

(57)

The present invention is directed to a methods and compositions for receptor mediated drug delivery, particularly across the blood-brain barrier.

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(54) Title: DELIVERY OF THERAPEUTIC COMPOUNDS TO THE BRAIN AND OTHER TISSUES

(57) Abstract: The present invention is directed to a methods and compositions for receptor mediated drug delivery, particularly across the blood-brain barrier.

**DELIVERY OF THERAPEUTIC COMPOUNDS TO THE BRAIN AND
OTHER TISSUES**

BACKGROUND

Field of the Invention

5 The present invention is generally directed to compositions for and methods for achieving the delivery of therapeutic and/or diagnostic/investigational agents.

Background of the Related Art

10 The brain is shielded against potentially harmful substances by the blood-brain barrier (BBB). The microvascular barrier between blood and brain is made up of a capillary endothelial layer surrounded by a basement membrane and tightly associated accessory cells (pericytes, astrocytes). The brain capillary endothelium is much less permeable to low-molecular weight solutes than other
15 capillary endothelia due to an apical band of tight association between the membranes of adjoining cells, referred to as tight junctions. In addition to diminished passive diffusion, brain capillary endothelia also exhibit less fluid-phase pinocytosis than other endothelial cells. Brain capillaries possess few fenestrae and few endocytic vesicles, compared to the capillaries of other organs (see Pardridge, J. Neurovirol. 5:
20 556-569, 1999). There is little transit across the BBB of large, hydrophilic molecules aside from some specific proteins such as transferrin, lactoferrin and low-density lipoproteins, which are taken up by receptor-mediated endocytosis (see Pardridge, J. Neurovirol. 5: 556-569, 1999); Tsuji and Tamai, Adv. Drug Deliv. Rev. 36: 277-290 (1999); Kusuhara and Sugiyama, Drug Discov. Today 6:150-156 (2001); Dehouck et
25 al. J. Cell. Biol. 138: 877-889 (1997); Fillebeen et al. J. Biol. Chem. 274: 7011-7017, 1999).

 The blood-brain barrier (BBB) also impedes access of beneficial active agents (e.g., therapeutic drugs and diagnostic agents) to central nervous system (CNS) tissues, necessitating the use of carriers for their transit. Blood-brain barrier
30 permeability is frequently a rate-limiting factor for the penetration of drugs or peptides into the CNS (see Pardridge, J. Neurovirol. 5: 556-569, 1999); Bickel et al., Adv. Drug Deliv. Rev. 46: 247-279, 2001). For example, management of the neurological manifestations of lysosomal storage diseases (LSDs) is significantly

impeded by the inability of therapeutic enzymes to gain access to brain cell lysosomes. LSDs are characterized by the absence or reduced activity of specific enzymes within cellular lysosomes, resulting in the accumulation of undegraded "storage material" within the intracellular lysosome, swelling and malfunction of the lysosomes, and ultimately cellular and tissue damage. Intravenous enzyme replacement therapy (ERT) is beneficial for LSDs (e.g. MPS I, MPS II). However, the BBB blocks the free transfer of many agents from blood to brain, and LSDs that present with significant neurological sequelae (e.g. MPS III, MLD, and GM1) are not expected to be as responsive to intravenous ERT. For such diseases, a method of delivering the replacement enzyme across the BBB and into the lysosomes of the affected cells would be highly desirable.

Three ways of circumventing the BBB to enhance brain delivery of an administered active agent include direct intra-cranial injection, transient permeabilization of the BBB, and modification of the active agent to alter tissue distribution. *Direct injection* of an active agent into brain tissue bypasses the vasculature completely, but suffers primarily from the risk of complications (infection, tissue damage) incurred by intra-cranial injections and poor diffusion of the active agent from the site of administration. *Permeabilization* of the BBB entails non-specifically compromising the BBB concomitant with injection of intravenous active agent and is accomplished through loosening tight junctions by hyperosmotic shock (e.g. intravenous mannitol). High plasma osmolarity leads to dehydration of the capillary endothelium with partial collapse of tight junctions, little selectivity in the types of blood-borne substances that gain access to the brain under these conditions, and damage over the course of a life-long regimen of treatment.

The distribution of an active agent into the brain may also be increased by transcytosis, the active transport of certain proteins from the luminal space (blood-side) to the abluminal space (brain-side) of the BBB. Transcytosis pathways are distinct from other vesicular traffic within the capillary endothelial cell and transit can occur without alteration of the transported materials. Transcytosis is a cell-type specific process mediated by receptors on the BBB endothelial surface. Attachment of an active agent to a transcytosed protein (vector or carrier) is expected to increase distribution of the active substance to the brain. In transcytosis, the vector is presumed to have a dominant effect on the distribution of the joined pair. Vector

proteins include antibodies directed at receptors on the brain capillary endothelium (Pardridge, J. Neurovirol. 5: 556-569, 1999) and ligands to such receptors (Fukuta et al., Pharm Res., 11(12):1681-8; 1994; Broadwell et al., Exp Neurol., 142(1):47-65 1996). Antibody vectors are transported through the capillary endothelium by a
5 process of adsorptive endocytosis (non-specific, membrane-phase endocytosis) and are far less efficiently transported than actual receptor ligands, which cross the BBB by a saturable, energy-dependent mechanism (Broadwell et al., Exp Neurol., 142(1):47-65 1996).

The lipoprotein receptor-related protein (LRP) receptor family
10 comprises a group of membrane-spanning, endocytic proteins with homology to the LDL receptor. Characterized as playing a key role in lipoprotein metabolism, LRP have subsequently been shown to bind a variety of ligands present in the blood. (Herz and Strickland, J Clin Invest., 108(6):779-84, 2001). LRP ligands include the lipoprotein-associated proteins ApoE, ApoJ and lipoprotein lipase; proteinases tPA,
15 uPA, Factor IX and MMP-9; proteinase inhibitors PAI-1, antithrombin III, alpha-2-macroglobulin and alpha-antitrypsin; the antibacterial protein lactoferrin; the chaperone receptor-associated protein (RAP), the hormone thyrotropin, the cofactor cobalamin and the lysosomal proteins saposin and sphingolipid activator protein. Four of these ligands, ApoJ (Zlokovic et al., Proc. Nat'l Acad. Sci., USA 93(9):4229-
20 34 1996; Zlokovic, Life Sci., 59(18):1483-97, 1996), thyrotropin (Marino et al., J. Biol. Chem., 275(10):7125-37 2000; Marino et al., J. Biol. Chem., 274(18):12898-904, 1999), lipoprotein lipase (Obunike et al. J. Biol. Chem., 276(12):8934-41, 2001) and cobalamin (Ramanujam et al., Arch Biochem Biophys., 315(1):8-15, 1994) have been shown to be transcytosed across capillary endothelial cells in vitro and in vivo
25 by LRP family members.

Taken together, the LRP receptor family comprises a pool of compositionally and functionally related receptors expressed at different levels in different tissues, including capillary endothelium, neurons and astrocytes. LRP family members are professional endocytic receptors that have also been shown to
30 transcytose ligands across polarized epithelia.

A unique LRP ligand is the receptor-associated protein, RAP, a 39kD chaperone localized to the endoplasmic reticulum and Golgi (Bu and Schwartz, Trends Cell. Biol. 8(7):272-6, 1998). RAP binds tightly to LRP in these

compartments preventing premature association of the receptor with co-expressed ligands (Herz and Willnow, Atherosclerosis 118 Suppl:S37-41, 1995). RAP serves as an attractive targeting sequence for LRP due to its high affinity for all members of the LRP receptor family (~2 nM) and ability to out-compete all known LRP ligands.

- 5 Since RAP is not secreted, endogenous levels in the blood are low. Endocytosis of RAP by LRP results in localization to the lysosome and complete degradation of the protein. Structure-function studies have been performed on RAP, providing some guidance on minimization of the sequence required to fulfill the targeting function (Melman, et al., J. Biol. Chem. 276(31): 29338-46, 2001). It is not known whether
- 10 RAP is transcytosed, but Megalin-RAP complexes have been shown to remain intact as far as the late endosome (Czekay et al., Mol. Biol. Cell. 8(3):517-32, 1997). The integrity of the Megalin-RAP complex through the Compartment of Uncoupling Ligand from Receptor (CURL) and into this late endosomal compartment is in contrast to the observed instability of other LRP-ligand complexes in the early
- 15 endosome. The LRP-RAP complex thus appears to have enhanced resistance to acid-dependent dissociation, a potential indicator of transcytotic competence. RAP could be engineered to be more specific for particular members of the LRP family. Such modifications would allow more selective targeting of RAP fusions to particular tissues, as dictated by the expression of different LRP family members on those
- 20 tissues.

Megalin

- Furthermore, RAP may be a suitable substitute for the mannose 6-phosphate targeting signal on lysosomal enzymes. The LRP-RAP system shares many features with the mannose-6-phosphate receptor (MPR)-mannose 6-phosphate (M6P) system: Both receptor-ligand complexes, LRP-RAP and MPR-M6P, exhibit
- 25 dissociation constants in the 1-2 nM range and are stable in the CURL. Both LRP and MPR are widely expressed on a variety of tissues and efficiently transport bound ligand to the lysosome. Both types of ligands are degraded upon reaching the lysosome. The advantage of RAP targeting over M6P targeting is that it depends on a protein sequence rather than a modified carbohydrate. Biosynthetic throughput and
- 30 quality control are much higher for an amino acid sequence than for a modified oligosaccharide, allowing for better drug yield, potency and safety. The LRP-RAP system may also provide a method of efficiently targeting other tissues. For example, the high density of the Very Low Density Lipoprotein Receptor (VLDLR), a member

of the LRP family), as well as LRP1 on muscle cells implies that RAP fusions could be taken up to a significant extent by muscle through LRP receptor-dependent endocytosis (Takahashi et al., Proc. Natl. Acad. Sci. U.S.A. 89(19):9252-6, 1992).

However, there remains a need for novel compounds, pharmaceutical compositions, and methods of administration of such compounds and compositions that can more effectively deliver active agents to the brain and other biological compartments. In particular, there is a need for such novel compounds, pharmaceutical compositions, and methods of administration which deliver active agents to the brain and tissues or organs that are set off from the blood compartment by capillary endothelial cells that are closely sealed by tight junctions. In particular, there is a need for such novel compounds, pharmaceutical compositions, and methods of administration, which efficiently target the delivery of an active agent to a wide variety of tissues. In particular, there is a need for such novel compounds, pharmaceutical compositions, and methods of administration, which target the delivery of an active agent to the lysosomal compartment of a cell within those tissues. This invention provides such compounds, pharmaceutical compositions and methods for their use.

|| wide range
of tissue

SUMMARY OF THE INVENTION

The present invention relates to the discovery that megalin ligands can be used as carriers or vectors for the delivery of active agents via transcytosis. An exemplary such ligand is RAP, which serves to increase the transport of therapeutic and /or diagnostic/investigational agents across the blood brain barrier and/or deliver agents to lysosomes of cells within and without the CNS.

In one aspect, the invention provides compounds comprising a megalin ligand or a megalin binding fragment of a megalin ligand conjugated to a therapeutic and/or diagnostic/investigational agent and pharmaceutical compositions of such compounds. In some embodiments, the megalin ligand or megalin binding fragment of such a ligand may be modified as desired to enhance its stability or pharmacokinetic properties (e.g., PEGylation of the RAP moiety of the conjugate, mutagenesis of the RAP moiety of the conjugate).

The present application specifically contemplates a compound comprising a megalin-binding moiety conjugated to an agent of interest. The agent typically may be selected from the group consisting of a therapeutic agent, a diagnostic agent, a marker of a disease of the central nervous system (CNS), a labeled monoclonal antibody which binds a marker of a CNS disorder. Therapeutic agents that are useful in the compounds contemplated herein include but are not limited to proteins, cytotoxic chemotherapeutic agents, protein nucleic acids, siRNA molecules, antisense molecule, and expression constructs that comprise a nucleic acid that encodes a therapeutic protein of interest. The megalin-binding moiety and the agent of interest may be directly linked to each other or alternatively may be linked through a linker, such as e.g., a peptide linker. Preferably, the megalin-binding moiety is a moiety that is transcytosed in vivo. Exemplary such moieties include but are not limited to RAP, thyroglobulin, lipoprotein lipase, lactoferrin, apolipoprotein J/clusterin, apolipoprotein B, apolipoprotein E, tissue type plasminogen activator, uPA, PAI-1, vitamin D-binding protein, vitamin A/retinol-binding protein, β 2-microglobulin, α 1-microglobulin, vitamin B12/cobalamin plasma carrier protein, transcobalamin (TC)-B12, PTH, insulin, EGF, prolactin, albumin, apo H, transthyretin, lysozyme, cytochrome-c, α -amylase, and Ca^{2+} , and aprotinin. The compound may optionally exclude ApoJ.

The invention contemplates a chimeric molecule for transcytotic delivery into the brain across the blood-brain barrier, the chimeric molecule comprising a megalin ligand conjugated to an active agent to be delivered across the blood-brain barrier by transcytosis, wherein the megalin ligand facilitates transport of the chimeric molecule across the blood-brain barrier. Also contemplated is a chimeric molecule for delivery into the brain by transcytosis across the blood-brain barrier, the chimeric molecule comprising an LRP ligand conjugated to an active agent to be delivered across the blood-brain barrier by transcytosis, wherein the megalin ligand binds preferentially to megalin as compared to LRP1. Any of the compounds or chimeric molecules contemplated herein may be prepared as pharmaceutical compositions comprising the compound or chimeric molecule in a pharmaceutically acceptable carrier, diluent or excipient.

In particular embodiments, the agent is a bioactive protein or peptide covalently linked to the megalin ligand or megalin binding fragment thereof. Such

conjugates or chimeric molecules may be formed by synthetic chemical reactions or joined by linker groups. In preferred embodiments, when the active agent is a protein or enzyme, the protein or enzyme is a human protein or enzyme, a fragment of the human protein or enzyme having a biological activity of a native protein or enzyme, or a polypeptide that has substantial amino acid sequence homology with the human protein or enzyme. In some embodiments, the agent is a protein of human or mammalian sequence, origin or derivation, in certain aspects, the protein forms a fusion protein with the megalin ligand or megalin binding fragment of such a ligand. The active agent polypeptide portion of the fusion protein may be a substance having therapeutic activity such as a growth factor, lymphokine or peptide drug. The agent may be an enzyme or other bioactive protein or polypeptide. In other embodiments, the agent is an enzyme or protein whose deficiency causes a human disease such as Pompe's disease (e.g. alpha-glucosidase). In other embodiments, the enzyme is selected for its beneficial effect. In other embodiments, the conjugate is formed by non-covalent bonds between the carrier and an antibody to which the active agent is attached.

The megalin ligand can also be of human or mammalian sequence origin or derivation. In preferred embodiments, the megalin ligand is selected from the group consisting of RAP, thyroglobulin, lipoprotein lipase, lactoferrin, apolipoprotein J/clusterin, apolipoprotein B, apolipoprotein E, tissue type plasminogen activator, uPA, PAI-1, vitamin D-binding protein, vitamin A/retinol-binding protein, β 2-microglobulin, α 1-microglobulin, vitamin B12/cobalamin plasma carrier protein, transcobalamin (TC)-B12, PTH, insulin, EGF, prolactin, albumin, apo H, transthyretin, lysozyme, cytochrome-c, α -amylase, and aprotinin.

In yet other embodiments of the invention, in each of its aspects, any of the above megalin ligands are identical to the amino acid sequence of the given ligand from a human or mammalian source. In other embodiments, the megalin ligand is the native protein from the human or mammal. In other embodiments, the RAP or RAP polypeptide is substantially homologous (i.e., at least 80%, 85%, 90%, 95%, 98%, or 99% identical in amino acid sequence) to the native protein over a length of at least 25, 50, 100, 150, or 200 amino acids, or the entire length of the megalin ligand.

In preferred embodiments, the megalin ligand is RAP or a megalin binding fragment of RAP. In other embodiments, the subject to which the conjugate is to be administered is human.

In a further aspect, the invention provides a method for delivering
5 therapeutic and/or diagnostic/investigational agents to the central nervous system using the megalin ligand/megalín receptor carrier system to transport such agents across the BBB formed by the capillary endothelial cells which are closely sealed by tight junctions. The invention thereby provides a novel route of administering agents with a site of action within the central nervous system. In a further embodiment, a
10 modulator of megalin is co-administered to modulate the therapeutic or adverse effects of such a conjugate.

The invention contemplates a method of delivering an agent into the central nervous system of an animal comprising administering the animal the agent conjugated to a megalin binding moiety, wherein the transport of the agent conjugated
15 to the megalin-binding moiety across the blood brain barrier of the animal is greater than the transport of the agent in the absence of conjugation to the megalin binding moiety. Also contemplated are methods of increasing transcytosis of an agent, comprising conjugating the agent to a megalin-binding moiety, wherein transcytosis of the agent when conjugated to the megalin-binding moiety is greater than the
20 transcytosis of the agent in the absence of the conjugation. The invention also contemplates treating a disorder in a mammal comprising administering to the animal a therapeutic agent conjugated to a megalin binding moiety. In the methods of the invention the megalin-binding moiety typically improves transcytosis of the therapeutic agent being delivered. Another method of the invention is for delivering a
25 therapeutic enzyme to a lysosomal compartment in a cell expressing megalin, comprising contacting the cell with a composition comprising the therapeutic enzyme conjugated to a megalin-binding moiety, wherein the uptake of the therapeutic enzyme into the lysosomal compartment of the cell is mediated through megalin present on the surface of the cell.

30 In some embodiments, the conjugated chimeric molecules which comprise a megalin ligand and an active agent comprise more than one therapeutic active agent useful in treating the same condition or disorder linked to a single megalin ligand. In some embodiments, from about 1 to about 5 or from 2 to 10

molecules of the active agent are attached to one megalin ligand molecule to be administered to a patient having the disease, condition or disorder.

In another aspect, the invention provides methods for using the megalin receptor-based delivery in the treatment of diseases, disorders, or conditions.

5 In one group of embodiments, the conjugates of active agents with a megalin ligand may be used to treat a CNS condition or disorder. In one group of particularly preferred embodiments to be treated, the CNS condition or disorder to be treated is a brain tumor or other neoplasia (e.g., a CNS tumor such as a glioblastoma). Such tumors or neoplasia may be primary tumors or may be metastases. In these

10 embodiments, the compounds according to the invention may comprise a megalin ligand or a megalin binding fragment of such a ligand conjugated to a cancer chemotherapeutic agent. Preferred compounds have from about 1 to about 20 molecules of the chemotherapeutic agent covalently linked to each megalin ligand moiety. Such compounds are excellent vehicles for enhanced delivery of

15 chemotherapeutic agents to brain tumors and other neoplasia localized in or around the brain, and for improved treatment of such tumors and neoplasia. In some embodiments, the cancer chemotherapeutic agents conjugated to a megalin ligand polypeptide may be the same or different. For instance, from 1 to 3 different chemotherapeutic agents may be attached in the same or a different moles megalin

20 ligand polypeptide per mole active agent ratio (e.g., 1:1; 1:2; 1:3; 1:4; and 1:5 to 1:10) with respect to the megalin ligand or megalin binding fragment of such a chimeric compound.

Preferred chemotherapeutic agents for such conjugates are cytotoxic chemotherapeutic agents and include, but are not limited to adriamycin, cisplatin, 5-fluorouracil, camptothecin, and paclitaxel. In another embodiment, the present

25 invention provides a method of treating a patient with a brain or CNS tumor or glioblastoma by administering to the patient a therapeutically effective amount of megalin ligand conjugated to the chemotherapeutic agent. In another embodiment, the present invention provides for a method for delivering a compound of interest

30 through the blood-brain barrier of a subject into the brain parenchyma where the compound is a chemotherapeutic able to interfere with the division of the tumor cells and are toxic for dividing cells. These compounds are liberated in the lysosomes

following degradation of the vector and can diffuse through the lysosomal membrane and enter the nucleus.

In another group of embodiments, the present invention provides compounds, pharmaceutical compositions, and methods for treating neurologic and psychiatric diseases and CNS diseases, disorders and conditions, including, but not limited to, Alzheimer's Disease, Parkinson's Disease, Multiple Sclerosis, and Amyotrophic Lateral Sclerosis. In some embodiments, the compounds of the invention comprise a megalin ligand polypeptide conjugated to a therapeutic agent for treating such diseases, disorders and conditions. In a preferred group of embodiments, the therapeutic agent is a peptide including, but not limited to, Nerve Growth Factor, other peptide hormones or growth factors, and peptide neurotransmitters. In another embodiment, the present invention provides for a method for delivering an active agent through the blood-brain barrier of a subject into the brain parenchyma where the active agent is a neurotrophic factors including, but not limited to, Nerve Growth Factor, Brain-Derived Neurotrophic Factor, Neurotrophin-3, Neurotrophin-4/5, aFGF, bFGF, CNTF, Leukaemia Inhibitory Factor, Cardiotrophin-1, TGFb, BMPs, GDFs, Neurturin, Artemin, Persephin, EGF, TGFa, Neuregulins, IGF-1, IGF-2, ADNF and PDGFs. Other factors such as caspase inhibitors can also be conjugated as the active agent member of the compound. In other embodiments, the active agent is a therapeutic antibody directed toward a constituent of the CNS. In other embodiments, the active agent is an antimicrobial agent for treating or preventing a CNS infection or an immunomodulator such as a lymphokine.

In some embodiments, the chimeric molecule that is a conjugate of a megalin ligand (or megalin binding fragment thereof, e.g., RAP or a megalin binding fragment of RAP) and an active agent is administered to treat a disease or condition selected from the group consisting of neurological diseases including, but not limited to, conditions such as Alzheimer's Disease, Parkinson's Disease, schizophrenia, and epilepsy; neurological cancers, such as primary brain tumors including glioma, meningioma, neurinoma, pituitary adenoma, medulloblastoma, craniopharyngioma, hemangioma, epidermoid, sarcoma and intracranial metastasis from other tumor sources, and neurological infections or neurological inflammatory conditions.

Other diseases of the brain also may be treated. Diseases of the brain fall into two general categories: (a) pathologic processes such as infections, trauma and neoplasm; and, (b) diseases unique to the nervous system which include diseases of myelin and degeneration of neurons. Brain-related degenerative diseases resulting from a decrease in neuronal survival include, for example, Alzheimer's disease, Parkinson's disease, Huntington's disease, ischemia-related disease and stroke, amyotrophic lateral sclerosis (ALS), spinal muscular atrophy, cerebellar degeneration. Demyelinating diseases include multiple sclerosis (MS) and its variants and perivenous encephalitis. Other diseases in which the principal pathologic change is primary demyelination, but which are usually classified in other categories include leukodystrophies such as metachromatic leukodystrophy due to deficiency of arylsulfatase A, Krabbe's disease due to deficiency of galactocerebroside beta-galactosidase, adrenoleukodystrophy and adrenomyeloneuropathy, and post-viral diseases such as progressive multifocal leukoencephalopathy, acute disseminated encephalomyelitis, acute necrotizing hemorrhagic leukoencephalitis. In addition there are mitochondrial encephalomyopathies. It is contemplated that the conjugates of the invention may be used in the treatment of such diseases.

In still other aspects, the megalin ligand conjugates of the invention can be used to treat non-CNS (i.e., non-BBB delimited diseases, such as cancers, diseases and conditions of non-CNS organs). For example, conjugated agents can be used to treat conditions affecting a patient's muscles.

In other aspects, the invention provides methods of treating tissues or organs having proportionately greater, preferably more than two-fold, amounts of megalin receptors on their cells than other tissues or organs. In still other aspects, the invention provides methods of treating tissues or organs having proportionately greater, preferably more than two-fold, amounts of LRP receptors on their cells than other tissues or organs. The selective biodistribution of megalin ligand-conjugated active agents can enhance the selective targeting of such conjugated agents to specific organs.

In a still further aspect, the invention provides a method for using the RAP/megalin carrier system in the diagnosis of diseases, disorders, or conditions. While it is taught herein that megalin is a preferred RAP binding receptor, it is contemplated that other receptors, e.g., LRP, could be used such that RAP/LRP

carrier systems may be used in the diagnosis of diseases, disorders or conditions. The present invention provides screening assays for identifying or selecting conjugates of megalin ligand (e.g., RAP) with active agents that can prevent, ameliorate, or treat a CNS disease or disorder by measuring the transcytosis of such agents in in vitro models or by measuring the ability of such conjugates to reach or bind to the brain parenchyma in vivo. Transcytosis or delivery can be assessed by labeling the conjugate and then monitoring or detecting the location or transport of the label in the test chamber for an in vitro method or in a tissue compartment(s) in an in vivo method. In addition, a therapeutic effect or other biological effect of the conjugate can be used to monitor for passage of the conjugate into the parenchyma of the central nervous system. In preferred embodiments, the CNS condition is a brain tumor.

In another aspect, the invention provides a method of delivering a therapeutic enzyme to a lysosome in a brain cell of a subject, comprising: (i) administering a compound comprising megalin ligand (or megalin binding fragment thereof) conjugated to the therapeutic enzyme, (ii) transporting such compound across the capillary endothelium; (iii) contact of such compound with an megalin receptor on the cell, thereby facilitating entry of such compound into such cell by endocytosis; and (iv) delivery to lysosomes within the cell. In certain other aspects, the invention provides such compounds, compositions, and methods for delivering a therapeutic agent or diagnostic agent to the lysosome of a cell.

In yet another aspect, the invention provides a megalin ligand (or megalin binding fragment thereof) conjugated to a therapeutic enzyme, and method of treating lysosomal storage diseases by administering such a conjugate, wherein the ligand-enzyme complex binds to megalin receptor and is transported across the cell membrane, enters the cell and is delivered to the lysosomes within the cell. In some embodiments, the invention also provides a method of treating a lysosomal storage disease in a patient by administering a megalin ligand (or megalin binding fragment thereof) conjugated to a therapeutic agent which is a protein or enzyme deficient in the lysosomes of a subject having such a disease (e.g., enzyme replacement therapy). Such conjugates are particularly useful, for example, in the treatment of lysosomal storage diseases such as MPS I, MPS II, MPS III A, MPS III B, Metachromatic Leukodystrophy, Gaucher, Krabbe, Pompe, CLN2, Niemann-Pick and Tay-Sachs disease wherein a lysosomal protein deficiency contributes to the disease state. In yet

other embodiments, the invention also provides a pharmaceutical composition comprising megalin ligand (e.g., RAP) covalently linked to a protein or enzyme deficient in a lysosomal storage disease.

Thus the invention contemplates methods of treating a lysosomal storage disease (LSD) in a subject comprising administering to the subject a composition comprising a megalin-binding moiety conjugated to a therapeutic agent used in the treatment of the LSD, in an amount effective to ameliorate the symptoms of the LSD. Typically, in such a method the composition is a pharmaceutical composition and is administered in an amount effective to decrease the amount of storage granules present in the brain tissue of the mammal. The administration may be intrathecal administration into the central nervous system of the mammal. Preferably, the composition is administered in an amount effective to decrease the amount of storage granules present in the meningeal tissue of the mammal. The symptoms of LSD are monitored using techniques known to those of skill in the art and are typically monitored through routine assessment of history, physical examination, echocardiography, electrocardiography, magnetic resonance imaging, polysomnography, skeletal survey, range of motion measurements, corneal photographs, and skin biopsy.

In some embodiments, the compounds, compositions, and methods of the invention can be used to treat such lysosomal storage diseases as Aspartylglucosaminuria, Cholesterol ester storage disease/Wolman disease, Cystinosis, Danon disease, Fabry disease, Farber Lipogranulomatosis/Farber disease, Fucosidosis, Galactosialidosis types I/II, Gaucher disease types I/II/III Gaucher disease, Globoid cell leukodystrophy/ Krabbe disease, Glycogen storage disease II/Pompe disease, GM1-Gangliosidosis types I/II/III, GM2-Gangliosidosis type I/Tay-Sachs disease, GM2-Gangliosidosis type II Sandhoff disease, GM2-Gangliosidosis, alpha-Mannosidosis types I/II, alpha-Mannosidosis, Metachromatic leukodystrophy, Mucopolipidosis type I/Sialidosis types I/II Mucopolipidosis types II /III I-cell disease, Mucopolipidosis type IIIC pseudo-Hurler polydystrophy, Mucopolysaccharidosis type I, Mucopolysaccharidosis type II Hunter syndrome, Mucopolysaccharidosis type IIIA Sanfilippo syndrome, Mucopolysaccharidosis type IIIB Sanfilippo syndrome, Mucopolysaccharidosis type IIIC Sanfilippo syndrome, Mucopolysaccharidosis type IIID Sanfilippo syndrome, Mucopolysaccharidosis type IVA Morquio syndrome,

Mucopolysaccharidosis type IVB Morquio syndrome, Mucopolysaccharidosis type VI, Mucopolysaccharidosis type VII Sly syndrome, Mucopolysaccharidosis type IX, Multiple sulfatase deficiency, Pompe, Neuronal Ceroid Lipofuscinosis, CLN1 Batten disease, Neuronal Ceroid Lipofuscinosis, CLN2 Batten disease, Niemann-Pick disease types A/B Niemann-Pick disease, Niemann-Pick disease type C1 Niemann-Pick disease, Niemann-Pick disease type C2 Niemann-Pick disease, Pycnodysostosis, Schindler disease types I/II Schindler disease, and Sialic acid storage disease. In particularly preferred embodiments, the lysosomal storage disease is MPS III, MLD, or GM1.

10 In still another embodiment, the present invention provides for a method of enzyme replacement therapy by administering a therapeutically effective amount of a conjugate to a subject in need of the enzyme replacement therapy, wherein the conjugate comprises a megalin ligand (or megalin binding fragment thereof) linked to an enzyme via a linker, wherein the cells of the patient have
15 lysosomes which contain insufficient amounts of the enzyme to prevent or reduce damage to the cells, whereby sufficient amounts of the enzyme enter the lysosomes to prevent or reduce damage to the cells. The cells may be within or without the CNS and may but need not be set off from the blood by capillary walls whose endothelial cells are closely sealed to diffusion of an active agent by tight junctions.

20 In some embodiments, the megalin ligand conjugates with an active agent comprise more than one active agent for treating a lysosomal storage disease linked to a single megalin ligand. In some embodiments, from about 1 to about 5 or from 2 to 10 molecules of the active agent of interest are bound to a single megalin ligand molecule. In preferred embodiments, the megalin ligand is RAP or a megalin-
25 binding fragment of a RAP polypeptide.

In a particular embodiment, the invention provides compounds comprising a megalin ligand bound to an active agent having a biological activity which is reduced, deficient, or absent in the target lysosome of the subject to which the compound is administered. In preferred embodiments, the megalin ligand (or
30 megalin binding fragment thereof) is covalently bound to the active agent. Preferred active agents include, but are not limited to aspartylglucosaminidase, acid lipase, cysteine transporter, Lamp-2, alpha-galactosidase A, acid ceramidase, alpha-L-fucosidase, beta-hexosaminidase A, GM2-activator deficiency, alpha-D-mannosidase,

beta-D-mannosidase, arylsulfatase A, saposin B, neuraminidase, alpha-N-acetylglucosaminidase phosphotransferase, phosphotransferase γ -subunit, alpha-L-iduronidase, iduronate-2-sulfatase, heparan-N-sulfatase, alpha-N-acetylglucosaminidase, acetylCoA:N-acetyltransferase, N-acetylglucosamine 6-sulfatase, galactose 6-sulfatase, alpha-galactosidase, N-acetylgalactosamine 4-sulfatase, hyaluronoglucosaminidase, palmitoyl protein thioesterase, tripeptidyl peptidase I, acid sphingomyelinase, cholesterol trafficking, cathepsin K, beta-galactosidase B, α -glucosidase, and sialic acid transporter. In a preferred embodiment, alpha-L-iduronidase, α -glucosidase or N-acetylgalactosamine 4-sulfatase is the enzyme.

In specific embodiments, the disease being treated by the methods provided herein mucopolysaccharidosis, more particularly, mucopolysaccharidosis I. In specific embodiments, the mammal with the LSD demonstrates about 50% or less of a normal α -L-iduronidase activity. Typically, the pharmaceutical composition is administered at a dose of between about 0.001mg/kg body weight and 0.5 mg/kg body weight of the human α -L-iduronidase administered weekly to a subject suffering from a deficiency thereof. These are merely exemplary and those of skill in the art may employ other doses to achieve therapeutically effective results. Further it should be understood that dosage form is cited herein as mg/kg body weight, however, those of skill in the art will be aware of other dosage measurements that may be used instead. In some embodiments, the pharmaceutical composition is administered at a dose of between about 0.01 mg/15 cc of CSF to about 5.0 mg/15 cc of CSF of the mammal of the human α -L-iduronidase is administered weekly to a subject suffering from a deficiency thereof. In the treatment of LSD, the administration of the megalin-binding moiety conjugated to a therapeutic agent preferably results in normalization of developmental delay and regression in the subject, reduction in high pressure hydrocephalus, reduction in spinal cord compression in the subject, and reduction in number and/or size of perivascular cysts around the brain vessels of the subject. Where the administration is intrathecal, such administration may comprise introducing the pharmaceutical composition into a cerebral ventricle. The methods may comprise intrathecal administration that introduces the pharmaceutical composition into the lumbar area or the cisterna magna. Intrathecal administration may be effected through the use of e.g., an infusion pump. It may be a continuous

administration over a period of time. Typically, the period of time may be at least several days. Preferably, the mammal being treated is a human.

Also contemplated is a method of promoting the breakdown of glycosaminoglycan (GAG) in a brain cell of a subject having lysosomal storage disease, the method comprising administering to the subject a pharmaceutical composition comprising an enzyme deficient in the lysosomal storage disease conjugated to a megalin-binding moiety in an amount effective to reduce the amount of GAG present in the brain cell as compared to the amount of GAG present in the cell prior to the administration. Preferably, the brain cell is a neuron, glial cell, microglial cell, astrocyte, oligodendroglial cell, perivascular cell, perithelial cell, meningeal cell, ependymal cell, arachnoid granulation cell, arachnoid membrane, dura mater, pia mater and choroid plexus cell. In these methods, the subject may manifest a symptom of high pressure hydrocephalus and the administering reduces the amount of CSF fluid in the meningeal tissue of the subject. In other aspects, the number of lysosomal storage granules in the cell are reduced as compared to the number of lysosomal storage granules present in a similar cell in the absence of administration of the conjugate. In other embodiments, the number of lysosomal storage granules in the cell are reduced as compared to the number of lysosomal storage granules present in a similar cell treated with enzyme alone without conjugation to the megalin-binding moiety.

In another aspect, the invention provides screening assays for identifying megalin ligand (or megalin binding fragment thereof) conjugated to active agent that can prevent, ameliorate, or treat a lysosomal storage disease by contacting a cell containing a lysosome with the conjugate and determining whether the conjugate delivers the agent to the lysosome. The delivery can be assessed by labeling the conjugate and then monitoring or detecting the location of the label in the cell or by determining the effect of the conjugate on the amount of the storage material found in the lysosome. In a preferred embodiment, the agent is a protein or enzyme deficient in the lysosomal storage disease. In another embodiment, the cell is deficient in the agent conjugated to the megalin ligand.

In another embodiment, the present invention provides for a method for identifying an agent that can prevent, ameliorate or treat a lysosomal storage disease, by administering megalin ligand (or megalin binding fragment thereof)

conjugated enzyme to a cell, wherein absence of the enzyme causes the lysosomal storage disease; and determining whether the agent reduces damage to the cell compared to damage to the cell if the conjugated agent was not administered to the cell. In certain embodiments, the method is a high throughput assay.

5 Other features and advantages of the invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, because various changes and modifications within the spirit and scope of the invention will become
10 apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE FIGURES

The following drawings form part of the present specification and are included to further illustrate aspects of the present invention. The invention may be
15 better understood by reference to the drawings in combination with the detailed description of the specific embodiments presented herein.

Figure 1. Effect of RAP on [¹²⁵I]-p97 transcytosis across BBCEC monolayers.

Figure 2. Preparation of expression constructs encoding fusions
20 between human RAP and human glucosidase (GAA), alpha-L-iduronidase (IDU) and glial-derived neurotrophic factor (GDNF). (RAPF primer: SEQ ID NO:12; RAPR primer SEQ ID NO:13; GAA forward primer SEQ ID NO:14; GAA reverse primer SEQ ID NO:15; IDU forward primer SEQ ID NO:16; IDU reverse primer SEQ ID NO:17; GDNF forward primer SEQ ID NO:18; GDNF reverse primer SEQ ID
25 NO:19; RAPBACF primer SEQ ID NO:20.)

Figure 3. Nucleotide and protein sequences of the RAP-GAA fusion (nucleotide sequence: SEQ ID NO:6; protein sequence: SEQ ID NO:7).

Figure 4. Nucleotide and protein sequence of RAP-IDU fusion (nucleotide sequence: SEQ ID NO:8; protein sequence: SEQ ID NO:9).

30 Figure 5. Nucleotide and protein sequence of RAP-GDNF fusion (nucleotide sequence: SEQ ID NO:10; protein sequence: SEQ ID NO:11).

Figure 6. Characterization of the RAP-GAA fusion.

Figure 7. Assay for complex oligosaccharides on RAP-GAA.

Figure 8. Assay for high-mannose oligosaccharides on RAP-GAA.

Figure 9. Characterization of RAP-IDU fusion.

5 Figure 10. Binding of RAP and RAP-lysosomal enzyme fusion to LRP.

Figure 11. Corrected Vd vs. perfusion time for iodinated RAP and transferrin at 15 minutes.

10 Figure 12. Distribution of RAP between brain capillary endothelium and brain parenchyma.

Figure 13. RAP-alpha-glucosidase uptake by human Pompe fibroblasts.

15 Figure 14. Multiple alignment of amino acid sequences of RAP from different species: human (SEQ ID NO:21); mouse (SEQ ID NO:22); rat (SEQ ID NO:23); chicken (SEQ ID NO:24); zebrafish (SEQ ID NO:25); fruit fly (SEQ ID NO:26); mosquito (SEQ ID NO:27); flatworm (SEQ ID NO:28).

Figure 15. SEQ ID NO:1, amino acid sequence of human RAP.

Figure 16. SEQ ID NO:2, amino acid sequence of the 28 kD RAP polypeptide.

20 Figure 17. Transcytosis in bovine brain capillary endothelial cells.

Figure 18. Transport of ¹²⁵I-RAP in MDCK cells showing basolateral-to-apical flux.

25 Figure 19A-19C. Figure 19A Gel and blot analysis of RAP fusions: RAP-IDU, A; RAP-GAA, B. Coomassie Blue staining, lane 1; anti-RAP antibody, lane 2; anti-IDU or anti-GAA antibody, lane 3. Figure 19B. Kinetic analysis of rhIDU and RAP-IDU: Proteins (1 nM) were incubated at room temperature for 5 minutes in different concentrations of 4-MUI. Derived V_{max} and K_m values are listed in Table A. Figure 19C. In vitro proteolysis of RAP fusions: Fusions were treated with a mixture of cathepsins, resolved on SDS-PAGE gels and stained with
30 Coomassie Blue. Undigested RAP-GAA fusion, lane 1; proteolyzed RAP-GAA

fusion, lane 2; rhGAA, lane 3; undigested RAP-IDU fusion, lane 4; proteolyzed RAP-IDU fusion, lane 5; rhIDU, lane 6; RAP, lane 7; molecular weight markers, lane 8.

Figure 20A-20C. Figure 20A. Fluorophore-assisted carbohydrate electrophoresis (FACE) of rhGAA (A), RAP-GAA (A), rhIDU (B) and RAP-IDU (B): N-linked oligosaccharides were released, fluorescently labeled at the reducing terminus and electrophoresed. Fluorescent bands were analyzed on a FACE imager system. Band intensity is proportional to the molar amount of particular oligosaccharides present. Oligoglucose ladder calibrated in degree of polymerization (DP) units, lane 1; rhGAA (A) or rhIDU (B), lane 2; RAP-GAA (A) or RAP-IDU (B), lane 3. The prominent band near the bottom of lane 2 in both A and B marked by the arrow is Bis-7. Figure 20B. Isoelectric focusing analysis of RAP-GAA for complex oligosaccharides: Proteins were treated with *Clostridium perfringens* neuraminidase, resolved on PhastGels and silver stained. Untreated rhIDU (positive control), lane 1; rhIDU treated with neuraminidase, lane 2; untreated RAP-GAA, lane 3; RAP-GAA treated with neuraminidase, lane 4; pI standards, lane 5. Figure 20C. Endo H and N-glycanase digestion of proteolyzed RAP-GAA and RAP-IDU: Fusions were proteolyzed with a mixture of cathepsins, treated with Endo H or N-glycanase, resolved on SDS-PAGE gels and stained with Coomassie Blue. Molecular weight standards, lanes 1 and 10. RAP-GAA, lane 2; proteolyzed RAP-GAA, lane 3; proteolyzed, endo H digested RAP-GAA, lane 4; proteolyzed, N-glycanase digested RAP-GAA, lane 5; RAP-IDU, lane 6; proteolyzed RAP-IDU, lane 7; proteolyzed, endo H digested RAP-IDU, lane 8; proteolyzed, N-glycanase digested RAP-IDU, lane 9. Interpolated molecular weights are printed under each band.

Figure 21. sLRP2 ligand blot: The second ligand-binding domain of LRP1 was blotted to nylon membrane and probed with ligands in the presence or absence of excess RAP. Bound ligands were detected by Western blotting with indicated antibodies. Ligands were: Buffer alone, column A; RAP, column B; RAP-IDU, column C; rhIDU, column D.

Figure 22A-22F. Figure 22A: Uptake of RAP-IDU and rhIDU into GM1391 fibroblasts: Different concentrations of proteins were incubated with fibroblasts for 2 hours. After washing, fibroblasts were lysed and uptake was measured by enzymatic assay. Curves were fitted and constants derived as described. Inset: Plot of rhIDU data alone. Figure 22B: Uptake of RAP-GAA and rhGAA into

GM244 fibroblasts: Different concentrations of proteins were incubated with fibroblasts for 2 hours. After washing, fibroblasts were lysed and uptake was measured by enzymatic assay. Curves were fitted and constants derived as described. Inset: Plot of rhGAA data alone. Figure 22C: Inhibition of RAP-IDU uptake into

5 GM1391 fibroblasts: RAP-IDU (3 nM) was incubated with fibroblasts in the presence of different concentrations of RAP for 2 hours. After washing, fibroblasts were lysed and uptake was measured by iduronidase enzymatic assay. Figure 22D: Inhibition of RAP-GAA uptake into GM244 fibroblasts: RAP-GAA (5 nM) was incubated with fibroblasts in the presence of different inhibitors for 2 hours. After

10 washing, fibroblasts were lysed and uptake was measured by enzymatic assay. Figure 22E. Inhibition of RAP-GAA (gray) and rhGAA (black) uptake into C6 glioma cells: Proteins (5 nM) were incubated with C6 glioma cells in the presence of inhibitors for 2 hours. After washing, fibroblasts were lysed and uptake was measured by enzymatic assay. ND = not done. Figure 22F Inhibition of RAP-GAA uptake (gray) and

15 rhGAA uptake (black) into C2C12 myoblasts: RAP-GAA and rhGAA, both at 5 nM, were incubated with cells in the presence of inhibitors for 2 hours. After washing, fibroblasts were lysed and uptake was measured by enzymatic assay.

Figure 23 RAP-GAA uptake mediated by different LRP receptors: Values represent the difference between uptake in the presence and absence of excess

20 cold RAP (receptor-specific uptake). Femtomoles of solubilized ^{125}I were normalized to total protein in each sample.

Figure 24 Intra-cellular half-life of RAP-GAA and rhGAA in GM244 fibroblasts: Proteins were incubated with fibroblasts for 24 hours. Medium was changed and cells were allowed to grow for intervals from 2 to 14 days, followed by

25 lysis and alpha-glucosidase enzyme assay.

Figure 25A-25B. Figure 25A. Clearance of stored glycosaminoglycan in Hurler fibroblasts by rhIDU and RAP-IDU: Cells were labeled in triplicate with ^{35}S -sulfate in the presence of rhIDU or RAP-IDU for 48 hours. Labeled cells were then washed, lysed and assayed for radioactivity and total protein. Figure 25B: SDS-

30 PAGE analysis of proteins used for experiment, stained with Coomassie Blue. RAP-IDU, lane 1; rhIDU, lane 2.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

Despite the fact that there have been significant advances made in the design and delivery of therapeutic agent across the blood brain barrier, there remains a need for new agents that may produce additional compounds that can mediate the transcytosis of therapeutic agents.

The present invention relates to the discovery that RAP and RAP polypeptides selectively bind to megalin receptors. Other embodiments are directed to exploiting findings that RAP or RAP polypeptides bind LRP receptors. RAP is a particularly effective megalin ligand for delivering active agents conjugated to it across the blood brain barrier, to the lysosomes within a cell, and to the intracellular compartment of cells bearing megalin receptors. Other megalin ligands also are exemplified herein as being useful in mediating such delivery. Compounds comprising megalin ligand (or megalin binding fragment thereof) conjugated to an active agent are useful in the diagnosis and treatment of a variety of CNS and non-CNS diseases, conditions, and disorders, including but not limited to, in particular, cancer and lysosomal storage diseases. Methods and compositions for exploiting these findings are described in further detail below.

I. DEFINITIONS

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. The following references provide one of skill with a general definition of many of the terms used in this invention: Singleton, *et al.*, DICTIONARY OF MICROBIOLOGY AND MOLECULAR BIOLOGY (2d ed. 1994); THE CAMBRIDGE DICTIONARY OF SCIENCE AND TECHNOLOGY (Walker ed., 1988); THE GLOSSARY OF GENETICS, 5TH ED., R. Rieger, *et al.* (eds.), Springer Verlag (1991); and Hale and Marham, THE HARPER COLLINS DICTIONARY OF BIOLOGY (1991).

Each publication, patent application, patent, and other reference cited herein is incorporated by reference in its entirety to the extent that it is not inconsistent with the present disclosure.

It is noted here that as used in this specification and the appended claims, the singular forms "a," "an," and "the" include plural reference unless the context clearly dictates otherwise.

As used herein, the following terms have the meanings ascribed to them unless specified otherwise.

“Brain tumors and other neoplasia in or around the brain” as used herein includes both primary tumors and/or metastases that develop in or around the brain. It may also mean metastases of brain tumors that migrate elsewhere in the body, but remain responsive to RAP or RAP polypeptide conjugates with chemotherapeutic agents. Many types of such tumors and neoplasia are known. Primary brain tumors include glioma, meningioma, neurinoma, pituitary adenoma, medulloblastoma, craniopharyngioma, hemangioma, epidermoid, sarcoma and others. Fifty percent of all intracranial tumors are intracranial metastasis. As used herein, tumors and neoplasia may be associated with the brain and neural tissue, or they may be associated with the meninges, skull, vasculature or any other tissue of the head or neck. Such tumors are generally solid tumors, or they are diffuse tumors with accumulations localized to the head. Tumors or neoplasia for treatment according to the invention may be malignant or benign, and may have been treated previously with chemotherapy, radiation and/or other treatments.

The term “effective amount” means a dosage sufficient to produce a desired result on a health condition, pathology, and disease of a subject or for a diagnostic purpose. The desired result may comprise a subjective or objective improvement in the recipient of the dosage. “Therapeutically effective amount” refers to that amount of an agent effective to produce the intended beneficial effect on health.

“Small organic molecule” refers to organic molecules of a size comparable to those organic molecules generally used in pharmaceuticals. The term excludes organic biopolymers (e.g., proteins, nucleic acids, etc.). Preferred small organic molecules range in size up to about 5000 Da, up to about 2000 Da, or up to about 1000 Da.

A “subject” of diagnosis or treatment is a human or non-human animal, including a mammal or a primate.

"Treatment" refers to prophylactic treatment or therapeutic treatment or diagnostic treatment.

A "prophylactic" treatment is a treatment administered to a subject who does not exhibit signs of a disease or exhibits only early signs for the purpose of decreasing the risk of developing pathology. The conjugate compounds of the invention may be given as a prophylactic treatment to reduce the likelihood of developing a pathology or to minimize the severity of the pathology, if developed.

A "therapeutic" treatment is a treatment administered to a subject who exhibits signs or symptoms of pathology for the purpose of diminishing or eliminating those signs or symptoms. The signs or symptoms may be biochemical, cellular, histological, functional, subjective or objective. The conjugate compounds of the invention may be given as a therapeutic treatment or for diagnosis.

"Diagnostic" means identifying the presence or nature of a pathologic condition. Diagnostic methods differ in their specificity and selectivity. While a particular diagnostic method may not provide a definitive diagnosis of a condition, it suffices if the method provides a positive indication that aids in diagnosis.

"Pharmaceutical composition" refers to a composition suitable for pharmaceutical use in subject animal, including humans and mammals. A pharmaceutical composition comprises a pharmacologically effective amount of a RAP polypeptide (or other megalin ligand) conjugated to an active agent and also comprises a pharmaceutically acceptable carrier. A pharmaceutical composition encompasses a composition comprising the active ingredient(s), and the inert ingredient(s) that make up the carrier, as well as any product which results, directly or indirectly, from combination, complexation or aggregation of any two or more of the ingredients, or from dissociation of one or more of the ingredients, or from other types of reactions or interactions of one or more of the ingredients. Accordingly, the pharmaceutical compositions of the present invention encompass any composition made by admixing a conjugate compound of the present invention and a pharmaceutically acceptable carrier.

"Pharmaceutically acceptable carrier" refers to any of the standard pharmaceutical carriers, buffers, and excipients, such as a phosphate buffered saline solution, 5% aqueous solution of dextrose, and emulsions, such as an oil/water or

water/oil emulsion, and various types of wetting agents and/or adjuvants. Suitable pharmaceutical carriers and formulations are described in Remington's Pharmaceutical Sciences, 19th Ed. (Mack Publishing Co., Easton, 1995). Preferred pharmaceutical carriers depend upon the intended mode of administration of the active agent. Typical modes of administration include enteral (e.g., oral) or parenteral (e.g., subcutaneous, intramuscular, intravenous or intraperitoneal injection; or topical, transdermal, or transmucosal administration). A "pharmaceutically acceptable salt" is a salt that can be formulated into a compound for pharmaceutical use including, e.g., metal salts (sodium, potassium, magnesium, calcium, etc.) and salts of ammonia or organic amines.

The term "unit dosage form," as used herein, refers to physically discrete units suitable as unitary dosages for human and animal subjects, each unit containing a predetermined quantity of compounds of the present invention calculated in an amount sufficient to produce the desired effect in association with a pharmaceutically acceptable diluent, carrier or vehicle. The specifications for the novel unit dosage forms of the present invention depend on the particular conjugate employed and the effect to be achieved, and the pharmacodynamics associated with each compound in the host.

"Modulate," as used herein, refers to the ability to alter, by increase or decrease (e.g., to act as an antagonist or agonist).

"Increasing relative delivery" as used herein refers to the effect whereby the accumulation at the intended delivery site (e.g., brain, lysosome) of a RAP-conjugated active agent is increased relative to the accumulation of the unconjugated active agent.

"Therapeutic index" refers to the dose range (amount and/or timing) above the minimum therapeutic amount and below an unacceptably toxic amount.

"Equivalent dose" refers to a dose, which contains the same amount of active agent.

"Polynucleotide" refers to a polymer composed of nucleotide units. Polynucleotides include naturally occurring nucleic acids, such as deoxyribonucleic acid ("DNA") and ribonucleic acid ("RNA") as well as nucleic acid analogs. Nucleic acid analogs include those which include non-naturally occurring bases, nucleotides

that engage in linkages with other nucleotides other than the naturally occurring phosphodiester bond or which include bases attached through linkages other than phosphodiester bonds. Thus, nucleotide analogs include, for example and without limitation, phosphorothioates, phosphorodithioates, phosphorotriesters, 5 phosphoramidates, boranophosphates, methylphosphonates, chiral-methyl phosphonates, 2-O-methyl ribonucleotides, peptide-nucleic acids (PNAs), and the like. Such polynucleotides can be synthesized, for example, using an automated DNA synthesizer. The term "nucleic acid" typically refers to large polynucleotides. The term "oligonucleotide" typically refers to short polynucleotides, generally no greater 10 than about 50 nucleotides. It will be understood that when a nucleotide sequence is represented by a DNA sequence (i.e., A, T, G, C), this also includes an RNA sequence (i.e., A, U, G, C) in which "U" replaces "T."

"cDNA" refers to a DNA that is complementary or identical to an mRNA, in either single stranded or double stranded form.

15 Conventional notation is used herein to describe polynucleotide sequences: the left-hand end of a single-stranded polynucleotide sequence is the 5'-end; the left-hand direction of a double-stranded polynucleotide sequence is referred to as the 5'-direction. The direction of 5' to 3' addition of nucleotides to nascent RNA transcripts is referred to as the transcription direction. The DNA strand having the 20 same sequence as an mRNA is referred to as the "coding strand"; sequences on the DNA strand having the same sequence as an mRNA transcribed from that DNA and which are located 5' to the 5'-end of the RNA transcript are referred to as "upstream sequences"; sequences on the DNA strand having the same sequence as the RNA and which are 3' to the 3' end of the coding RNA transcript are referred to as "downstream 25 sequences."

"Complementary" refers to the topological compatibility or matching together of interacting surfaces of two polynucleotides. Thus, the two molecules can be described as complementary, and furthermore, the contact surface characteristics are complementary to each other. A first polynucleotide is complementary to a 30 second polynucleotide if the nucleotide sequence of the first polynucleotide is identical to the nucleotide sequence of the polynucleotide binding partner of the second polynucleotide. Thus, the polynucleotide whose sequence 5'-TATAC-3' is complementary to a polynucleotide whose sequence is 5'-GTATA-3'.

A nucleotide sequence is "substantially complementary" to a reference nucleotide sequence if the sequence complementary to the subject nucleotide sequence is substantially identical to the reference nucleotide sequence.

"Encoding" refers to the inherent property of specific sequences of nucleotides in a polynucleotide, such as a gene, a cDNA, or an mRNA, to serve as templates for synthesis of other polymers and macromolecules in biological processes having either a defined sequence of nucleotides (i.e., rRNA, tRNA and mRNA) or a defined sequence of amino acids and the biological properties resulting therefrom. Thus, a gene encodes a protein if transcription and translation of mRNA produced by that gene produces the protein in a cell or other biological system. Both the coding strand, the nucleotide sequence of which is identical to the mRNA sequence and is usually provided in sequence listings, and non-coding strand, used as the template for transcription, of a gene or cDNA can be referred to as encoding the protein or other product of that gene or cDNA. Unless otherwise specified, a "nucleotide sequence encoding an amino acid sequence" includes all nucleotide sequences that are degenerate versions of each other and that encode the same amino acid sequence. Nucleotide sequences that encode proteins and RNA may include introns.

"Recombinant polynucleotide" refers to a polynucleotide having sequences that are not naturally joined together. An amplified or assembled recombinant polynucleotide may be included in a suitable vector, and the vector can be used to transform a suitable host cell. A host cell that comprises the recombinant polynucleotide is referred to as a "recombinant host cell." The gene is then expressed in the recombinant host cell to produce, e.g., a "recombinant polypeptide." A recombinant polynucleotide may serve a non-coding function (e.g., promoter, origin of replication, ribosome-binding site, etc.) as well.

"Expression control sequence" refers to a nucleotide sequence in a polynucleotide that regulates the expression (transcription and/or translation) of a nucleotide sequence operatively linked thereto. "Operatively linked" refers to a functional relationship between two parts in which the activity of one part (e.g., the ability to regulate transcription) results in an action on the other part (e.g., transcription of the sequence). Expression control sequences can include, for example and without limitation, sequences of promoters (e.g., inducible or constitutive),

enhancers, transcription terminators, a start codon (i.e., ATG), splicing signals for introns, and stop codons.

"Expression vector" refers to a vector comprising a recombinant polynucleotide comprising expression control sequences operatively linked to a nucleotide sequence to be expressed. An expression vector comprises sufficient cis-
5 acting elements for expression; other elements for expression can be supplied by the host cell or in vitro expression system. Expression vectors include all those known in the art, such as cosmids, plasmids (e.g., naked or contained in liposomes) and viruses that incorporate the recombinant polynucleotide.

10 "Amplification" refers to any means by which a polynucleotide sequence is copied and thus expanded into a larger number of polynucleotide molecules, e.g., by reverse transcription, polymerase chain reaction, and ligase chain reaction.

"Primer" refers to a polynucleotide that is capable of specifically
15 hybridizing to a designated polynucleotide template and providing a point of initiation for synthesis of a complementary polynucleotide. Such synthesis occurs when the polynucleotide primer is placed under conditions in which synthesis is induced, i.e., in the presence of nucleotides, a complementary polynucleotide template, and an agent for polymerization such as DNA polymerase. A primer is typically single-stranded,
20 but may be double-stranded. Primers are typically deoxyribonucleic acids, but a wide variety of synthetic and naturally occurring primers are useful for many applications. A primer is complementary to the template to which it is designed to hybridize to serve as a site for the initiation of synthesis, but need not reflect the exact sequence of the template. In such a case, specific hybridization of the primer to the template
25 depends on the stringency of the hybridization conditions. Primers can be labeled with, e.g., chromogenic, radioactive, or fluorescent moieties and used as detectable moieties.

"Probe," when used in reference to a polynucleotide, refers to a polynucleotide that is capable of specifically hybridizing to a designated sequence of
30 another polynucleotide. A probe specifically hybridizes to a target complementary polynucleotide, but need not reflect the exact complementary sequence of the template. In such a case, specific hybridization of the probe to the target depends on

the stringency of the hybridization conditions. Probes can be labeled with, e.g., chromogenic, radioactive, or fluorescent moieties and used as detectable moieties.

A first sequence is an "antisense sequence" with respect to a second sequence if a polynucleotide whose sequence is the first sequence specifically
5 hybridizes with a polynucleotide whose sequence is the second sequence.

"Hybridizing specifically to" or "specific hybridization" or "selectively hybridize to", refers to the binding, duplexing, or hybridizing of a nucleic acid molecule preferentially to a particular nucleotide sequence under stringent conditions when that sequence is present in a complex mixture (e.g., total cellular) DNA or
10 RNA.

The term "stringent conditions" refers to conditions under which a probe will hybridize preferentially to its target subsequence, and to a lesser extent to, or not at all to, other sequences. "Stringent hybridization" and "stringent hybridization wash conditions" in the context of nucleic acid hybridization
15 experiments such as Southern and Northern hybridizations are sequence dependent, and are different under different environmental parameters. An extensive guide to the hybridization of nucleic acids is found in Tijssen (1993) Laboratory Techniques in Biochemistry and Molecular Biology--Hybridization with Nucleic Acid Probes part I chapter 2 "Overview of principles of hybridization and the strategy of nucleic acid
20 probe assays", Elsevier, New York. Generally, highly stringent hybridization and wash conditions are selected to be about 5°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. The T_m is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe. Very stringent conditions are
25 selected to be equal to the T_m for a particular probe.

An example of stringent hybridization conditions for hybridization of complementary nucleic acids which have more than 100 complementary residues on a filter in a Southern or northern blot is 50% formalin with 1 mg of heparin at 42°C, with the hybridization being carried out overnight. An example of highly stringent
30 wash conditions is 0.15 M NaCl at 72°C for about 15 minutes. An example of stringent wash conditions is a 0.2X SSC wash at 65°C for 15 minutes (see, Sambrook et al. for a description of SSC buffer). Often, a high stringency wash is preceded by a

low stringency wash to remove background probe signal. An example medium stringency wash for a duplex of, e.g., more than 100 nucleotides, is 1x SSC at 45°C for 15 minutes. An example low stringency wash for a duplex of, e.g., more than 100 nucleotides, is 4-6x SSC at 40°C for 15 minutes. In general, a signal to noise ratio of
5 2x (or higher) than that observed for an unrelated probe in the particular hybridization assay indicates detection of a specific hybridization.

"Polypeptide" refers to a polymer composed of amino acid residues, related naturally occurring structural variants, and synthetic non-naturally occurring analogs thereof linked via peptide bonds, related naturally occurring structural
10 variants, and synthetic non-naturally occurring analogs thereof. Synthetic polypeptides can be synthesized, for example, using an automated polypeptide synthesizer. The term "protein" typically refers to large polypeptides. The term "peptide" typically refers to short polypeptides.

Conventional notation is used herein to portray polypeptide sequences:
15 the left-hand end of a polypeptide sequence is the amino-terminus; the right-hand end of a polypeptide sequence is the carboxyl-terminus.

- 1) Alanine (A), Serine (S), Threonine (T);
- 2) Aspartic acid (D), Glutamic acid (E);
- 3) Asparagine (N), Glutamine (Q);
- 20 4) Arginine (R), Lysine (K);
- 5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); and
- 6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W).

"Allelic variant" refers to any of two or more polymorphic forms of a gene occupying the same genetic locus. Allelic variations arise naturally through
25 mutation, and may result in phenotypic polymorphism within populations. Gene mutations can be silent (no change in the encoded polypeptide) or may encode polypeptides having altered amino acid sequences. "Allelic variants" also refer to cDNAs derived from mRNA transcripts of genetic allelic variants, as well as the proteins encoded by them.

30 The terms "identical" or percent "identity," in the context of two or more polynucleotide or polypeptide sequences, refer to two or more sequences or

subsequences that are the same or have a specified percentage of nucleotides or amino acid residues that are the same, when compared and aligned for maximum correspondence, as measured using one of the following sequence comparison algorithms or by visual inspection.

5 The phrase "substantially homologous" or "substantially identical" in the context of two nucleic acids or polypeptides, generally refers to two or more sequences or subsequences that have at least 40%, 60%, 80%, 90%, 95%, 98% nucleotide or amino acid residue identity, when compared and aligned for maximum correspondence, as measured using one of the following sequence comparison
10 algorithms or by visual inspection. Preferably, the substantial identity exists over a region of the sequences that is at least about 50 residues in length, more preferably over a region of at least about 100 residues, and most preferably the sequences are substantially identical over at least about 150 residues. In a most preferred embodiment, the sequences are substantially identical over the entire length of either
15 or both comparison biopolymers.

For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are input into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters
20 are designated. The sequence comparison algorithm then calculates the percent sequence identity for the test sequence(s) relative to the reference sequence, based on the designated program parameters.

Optimal alignment of sequences for comparison can be conducted, e.g., by the local homology algorithm of Smith and Waterman, Adv. Appl. Math.
25 2:482 (1981), by the homology alignment algorithm of Needleman and Wunsch, J. Mol. Biol. 48:443 (1970), by the search for similarity method of Pearson and Lipman, Proc. Natl. Acad. Sci. USA 85:2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by
30 visual inspection.

One example of a useful algorithm is PILEUP. PILEUP creates a multiple sequence alignment from a group of related sequences using progressive,

pairwise alignments to show relationship and percent sequence identity. It also plots a tree or dendrogram showing the clustering relationships used to create the alignment. PILEUP uses a simplification of the progressive alignment method of Feng and Doolittle, *J. Mol. Evol.* 35:351-360 (1987). The method used is similar to the method described by Higgins and Sharp, *CABIOS* 5:151-153 (1989). The program can align up to 300 sequences, each of a maximum length of 5,000 nucleotides or amino acids. The multiple alignment procedure begins with the pairwise alignment of the two most similar sequences, producing a cluster of two aligned sequences. This cluster is then aligned to the next most related sequence or cluster of aligned sequences. Two clusters of sequences are aligned by a simple extension of the pairwise alignment of two individual sequences. The final alignment is achieved by a series of progressive, pairwise alignments. The program is run by designating specific sequences and their amino acid or nucleotide coordinates for regions of sequence comparison and by designating the program parameters. For example, a reference sequence can be compared to other test sequences to determine the percent sequence identity relationship using the following parameters: default gap weight (3.00), default gap length weight (0.10), and weighted end gaps. Another algorithm that is useful for generating multiple alignments of sequences is Clustal W (Thompson et al. *Nucleic Acids Research* 22: 4673-4680, 1994).

Another example of algorithm that is suitable for determining percent sequence identity and sequence similarity is the BLAST algorithm, which is described in Altschul et al., *J. Mol. Biol.* 215:403-410 (1990). Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>). This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold. These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are then extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always > 0) and N (penalty score for mismatching residues; always < 0). For amino

acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring
5 residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, an expectation (E) of 10, M=5, N=4, and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a wordlength (W) of 3, an
10 expectation (E) of 10, and the BLOSUM62 scoring matrix (see Henikoff and Henikoff, Proc. Natl. Acad. Sci. USA 89:10915, 1989).

In addition to calculating percent sequence identity, the BLAST algorithm also performs a statistical analysis of the similarity between two sequences (see, e.g., Karlin and Altschul, Proc. Nat'l. Acad. Sci. USA 90:5873-5787, 1993).
15 One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a nucleic acid is considered similar to a reference sequence if the smallest sum probability in a comparison of the test nucleic acid to the reference nucleic acid
20 is less than about 0.1, more preferably less than about 0.01, and most preferably less than about 0.001.

A further indication that two nucleic acid sequences or polypeptides are substantially identical is that the polypeptide encoded by the first nucleic acid is immunologically cross reactive with the polypeptide encoded by the second nucleic
25 acid, as described below. Thus, a polypeptide is typically substantially identical to a second polypeptide, for example, where the two peptides differ only by conservative substitutions. Another indication that two nucleic acid sequences are substantially identical is that the two molecules hybridize to each other under stringent conditions, as described herein.

30 "Substantially pure" or "isolated" means an object species is the predominant species present (i.e., on a molar basis, more abundant than any other individual macromolecular species in the composition), and a substantially purified fraction is a composition wherein the object species comprises at least about 50% (on

a molar basis) of all macromolecular species present. Generally, a substantially pure composition means that about 80% to 90% or more of the macromolecular species present in the composition is the purified species of interest. The object species is purified to essential homogeneity (contaminant species cannot be detected in the composition by conventional detection methods) if the composition consists essentially of a single macromolecular species. Solvent species, small molecules (<500 Daltons), stabilizers (e.g., BSA), and elemental ion species are not considered macromolecular species for purposes of this definition. In some embodiments, the conjugates of the invention are substantially pure or isolated. In some embodiments, the conjugates of the invention are substantially pure or isolated with respect to the macromolecular starting materials used in their synthesis. In some embodiments, the pharmaceutical composition of the invention comprises a substantially purified or isolated conjugate of a RAP polypeptide and the active agent admixed with one or more pharmaceutically acceptable excipient.

"Naturally-occurring" as applied to an object refers to the fact that the object can be found in nature. For example, a polypeptide or polynucleotide sequence that is present in an organism (including viruses) that can be isolated from a source in nature and which has not been intentionally modified by man in the laboratory is naturally-occurring.

"Detecting" refers to determining the presence, absence, or amount of an analyte in a sample, and can include quantifying the amount of the analyte in a sample or per cell in a sample.

"Detectable moiety" or a "label" refers to a composition detectable by spectroscopic, photochemical, biochemical, immunochemical, or chemical means. For example, useful labels include ^{32}P , ^{35}S , fluorescent dyes, electron-dense reagents, enzymes (e.g., as commonly used in an ELISA), biotin-streptavidin, dioxigenin, haptens and proteins for which antisera or monoclonal antibodies are available, or nucleic acid molecules with a sequence complementary to a target. The detectable moiety often generates a measurable signal, such as a radioactive, chromogenic, or fluorescent signal, that can be used to quantitate the amount of bound detectable moiety in a sample. The detectable moiety can be incorporated in or attached to a primer or probe either covalently, or through ionic, van der Waals or hydrogen bonds, e.g., incorporation of radioactive nucleotides, or biotinylated nucleotides that are

recognized by streptavidin. The detectable moiety may be directly or indirectly detectable. Indirect detection can involve the binding of a second directly or indirectly detectable moiety to the detectable moiety. For example, the detectable moiety can be the ligand of a binding partner, such as biotin, which is a binding partner for streptavidin, or a nucleotide sequence, which is the binding partner for a complementary sequence, to which it can specifically hybridize. The binding partner may itself be directly detectable, for example, an antibody may be itself labeled with a fluorescent molecule. The binding partner also may be indirectly detectable, for example, a nucleic acid having a complementary nucleotide sequence can be a part of a branched DNA molecule that is in turn detectable through hybridization with other labeled nucleic acid molecules. (See, e.g., PD. Fahrlander and A. Klausner, *Bio/Technology* (1988) 6:1165.) Quantitation of the signal is achieved by, e.g., scintillation counting, densitometry, or flow cytometry.

"Linker" refers to a molecule that joins two other molecules, either covalently, or through ionic, van der Waals or hydrogen bonds, e.g., a nucleic acid molecule that hybridizes to one complementary sequence at the 5' end and to another complementary sequence at the 3' end, thus joining two non-complementary sequences.

II. MEGALIN

Megalin, also referred to as LRP2 is a large (600 kDa), is a member of the LRP family of receptors (Hussain et al. *Annu Rev Nutr.*, 19:141-72 1999; Christensen and Birn *Am. J. Physiol. Renal. Physiol.*, 280:F562-573, 2001). Like all members of the LRP family, megalin binds RAP with high affinity (Czekay et al., *Mol. Biol. Cell.* 8(3):517-32, 1997). Unique among the LRP family, however, megalin is expressed only on the apical surface of a restricted set of epithelial cell layers, including those in the kidney proximal tubule, the thyroid, the epididymis, the alveolae and the ciliary body of the eye (Zheng et al. *J Histochem Cytochem.*, 42(4):531-42, 1994). Megalin is also expressed on the luminal surface of the brain capillary endothelium, a classical squamous epithelial cell layer (Chun, et al. *Exp Neurol.*, 157(1):194-201, 1999). Megalin on the brain capillary endothelium has been previously demonstrated to mediate transcytosis of one of its ligands, apoJ, across the

blood-brain barrier in vitro (Zlokovic et al., Proc. Nat'l Acad. Sci., USA 93(9):4229-34 1996; Zlokovic Life Sci., 59(18):1483-97, 1996). Apical-to-basolateral transcytosis of ligands by megalin has also been documented in the kidney and thyroid (Marino et al. J Am Soc Nephrol., 12(4):637-48, 2001; Marino et al., Thyroid, 5 11(1):47-56, 2001).

In the present application it is shown that megalin mediates the transcytosis of RAP across tight MDCK cell layers. This application for the first time shows that it is megalin rather than LRP1 that mediates the transcytosis of RAP and other ligands across such cell layers. Given this finding, it is contemplated that the use of any and all megalin ligands will be excellent candidates for mediating the 10 delivery of active agents through targeted delivery in the kidney, thyroid, epididymis, eye and brain cells. Thus, in particular embodiments, while it remains desirable to use other LRP family members to mediate transcytosis of an active agent across the BBB, in particularly preferred embodiments, such transcytosis is mediated through 15 the conjugation of the active agent to a megalin ligand.

Thus, the present application contemplates that ligands with enhanced specificity for megalin over LRP1 will be particularly useful as vectors for the transport of proteins and small molecules from blood-to-brain. In certain embodiments, the ligands optionally exclude ApoJ. This advantage accrues from 20 avoiding LRP1-mediated clearance in the liver, increasing serum residence time and, consequently, brain influx.

III. OTHER LRP RECEPTORS

While megalin is the preferred receptor through which active agent transcytosis is achieved, it is contemplated that other LRP receptors will nonetheless 25 be useful for effecting such transcytosis. "LRP" refers to members of the low-density lipoprotein receptor family including the low-density lipoprotein receptor-related protein 1 (LRP1). LRP1 is a large protein of 4525 amino acids (600 kDa), which is cleaved by furin to produce two subunits of 515-(alpha) kD and 85-(B) kDa that 30 remain non-covalently bound. LRP is expressed on most tissue types. Other members of the low-density lipoprotein (LDL) receptor family include LDL-R (132 kDa); LRP/LRP1 and LRP1B (600 kDa); Megalin ((LRP2), 600 kDa); VLDL-R (130

kDa); ER-2 (LRP-8, 130 kDa); Mosaic LDL-R (LR11, 250 KDa); and other members such as LRP3, LRP6, and LRP-7. Characteristic features of the family include cell-surface expression; extracellular ligand binding domain repeats (DxSDE); requirement of Ca++ for ligand binding; recognition of RAP and ApoE; EGF precursor homology domain repeats (YWTD); single membrane spanning region; internalization signals in the cytoplasmic domain (FDNPXY); and receptor mediated endocytosis of various ligands. Some members of the family, including LRP1 and VLDLR, participate in signal transduction pathways.

LRP ligands refer to a number of molecules that are known to bind LRP. These molecules include, for instance, lactoferrin, RAP, lipoprotein lipase, ApoE, Factor VIII, beta-amyloid precursor, alpha-2-macroglobulin, thrombospondin 2 MMP-2 (matrix metalloproteinase-2), MPP-9-TIMP-1 (tissue inhibitor of matrix metalloproteinase-1); uPA (urokinase plasminogen activator):PAI-I (plasminogen activator inhibitor-1):uPAR (uPA receptor); and tPA (tissue plasminogen activator):PAI-1:uPAR.

LRP1 is believed to be a multifunctional receptor with clustering of cysteine-rich type repeats. A binding repeat, resembling those found in the LDL receptor, is the molecular principle for the ability to bind a variety of ligands that were previously thought to be unrelated. These include the ligands described in the previous paragraph in addition to: pseudomonas exotoxin A, human rhinovirus, lactoferrin and the so-called receptor associated protein (RAP). See, Meilinger et al., FEBS Lett, 360:70-74 (1995). LRP1 has the GenBank Accession No.: X 13916 and SwissProt Primary Accession No.: Q07954. Alternative names for the LRP1 gene/protein include: Low-density lipoprotein receptor-related protein 1 [precursor], LRP, Alpha-2-macroglobulin receptor, A2MR, Apolipoprotein E receptor, ApoER, CD91, LRP1 or A2MR.

Members of the LRP family are well expressed on capillary endothelium and on CNS cell types including neurons and astrocytes (e.g., LDL receptor, Megalin, LRP). LRP receptors endocytose bound ligand and have been demonstrated to transcytose ligands across polarized epithelial cells in the kidney, thyroid and across capillary endothelial cells in the brain. LRP therefore comprises a pool of compositionally and functionally related receptors expressed at different levels in different tissues. In some embodiments, this invention uses RAP, which

binds and thereby targets members of this pool of related receptors (and particularly cells, tissues, and organs expressing a member of this pool). Examples include the VLDLR on muscle tissue, LRP1B on neuronal tissue, Megalin on both kidney and neuronal tissue and LRP1 on vascular smooth muscle tissue.

5

IV. RAP AND OTHER MEGALIN LIGANDS

In specific embodiments of the present invention chimeric molecules are made, which comprise first portion that is a megalin ligand or a megalin binding fragment thereof and a second portion that is an active agent whose delivery will be mediated through the binding of the megalin ligand (or fragment thereof) to megalin. In preferred embodiments, the ligand selected to form part of these chimeric molecules will be one which is transcytosed in vivo. "RAP" is a well-known protein of about 39 kDa and 323 amino acids and is a specialized chaperone for members of the LRP family. It is transcytosed in vivo. RAP inhibits the binding of ligand to members of the LDL-receptor family such as LRP (see Bu and Rennke, J. Biol. Chem. 271: 22218-2224 (1996); Willnow et al., J. Biol. Chem. 267: 26172-26180 (1992); Bu and Schwartz, Trends Cell Biol. 8: 272-276 (1998); and Herz and Strickland, J. Clin. Invest. 108: 779-784 (2001). See also, Bu and Schwartz, Trends Cell Biol. 8: 272-276 (1998). Further characterization of RAP, including the complete amino acid sequence of human RAP (Figure 15), is found in U.S. Patent No. 5,474,766 which is incorporated herein by reference in its entirety and also with particularity with respect to the RAP amino acid sequences and fragments disclosed therein. The 28 kDa human C-terminal fragment (Figure 16) is an extremely active RAP polypeptide and in preferred embodiments of the invention, the conjugate comprises this fragment as the carrier for the active agent.

RAP polypeptides include, but are not limited to, RAP, soluble forms of RAP, cleaved RAP, RAP polypeptide fragments, homologues and analogs of RAP, and the like. RAP polypeptides that are functional equivalents of RAP with respect to modulation of LRP receptor binding, transcytosis, or endocytosis can be readily identified by screening for the ability of the RAP polypeptide to bind to LRP. In preferred embodiments, the RAP polypeptide is a homologue of RAP having, for instance, greater than 80%, 90% 95%, 98%, or 99% sequence identity with a naturally

occurring, native or wild type mammalian RAP amino acid sequence of similar length or over a domain of at least 10 amino acids, 25 amino acids, 50 amino acids, 100 amino acids, or 200 amino acids, 300 amino acids, or the entire length of the RAP polypeptide. RAP polypeptides include allelic variants of RAP, paralogs and
5 orthologs in human, mouse, rat, chicken, zebrafish, pig, fruit fly, mosquito, and flatworm native RAP, and derivatives, portions, or fragments thereof (Genbank accession numbers: P30533 (human), XP132029 (mouse), Q99068 (rat), CAA05085 (chicken), AAH49517 (zebrafish), AAM90301 (pig), NP649950 (fruit fly), XP313261 (mosquito), NP506187 (flatworm). A multiple alignment of amino acid sequences
10 from mouse, rat, chicken zebrafish, fruitfly, mosquito, and flatworm and the consensus sequence is shown in Figure 14.

The RAP polypeptide can be in the form of acidic or basic salts, or in its neutral form. In addition, individual amino acid residues can be modified, such as by oxidation or reduction. Moreover, various substitutions, deletions, or additions can
15 be made to the amino acid or nucleic acid sequences, the net effect of which is to retain or improve upon the desired biological activity of RAP. Further characterization of RAP, including the complete amino acid sequence of RAP, is found in U.S. Patent No. 5,474,766 which is incorporated herein by reference in its entirety and also with particularity with respect to the amino acid sequences of the
20 various RAP polypeptides disclosed therein. Due to code degeneracy, for example, one of ordinary skill in the art would know of considerable variations of the nucleotide sequences encoding the same amino acid sequence.

Preferred RAP polypeptides share substantial homology with the native amino acid sequence of a receptor associated protein (RAP), particularly the
25 native human sequence (SEQ ID NO:1). In preferred embodiments, the RAP polypeptide is a homologue of RAP having, for instance, greater than 80%, 90% 95%, 98%, or 99% sequence identity with a native or wild type mammalian RAP amino acid sequence of similar length or over a domain or comparison window of at least 10, amino acids, 25 amino acids, 50 amino acids, 100 amino acids, or 200 amino acids, or
30 300 amino acids or more.

An especially preferred human or mammalian RAP is isolated RAP or a fragment thereof, such as a soluble polypeptide fragment of RAP, which contains at least one of the RAP binding sites for LRP. Substantial guidance exists in the art to

which portions of RAP are important to its LRP binding and modulatory activity and which portions may be mutated, altered, or deleted without loss of binding activity (see, Nielsen et al. Proc. Nat. Acad. Sci. USA 94:7521 (1997); and Rall et al. J. Biol. Chem. 273(37):24152, 1998). For instance, RAP's LRP binding function has been mapped by performing direct binding studies on fusion proteins representing overlapping domains of RAP (see Willnow et al., J. Biol. Chem. 267(36):26172-80, 1992). The RAP binding motifs have also been characterized by use of truncated and site-directed RAP mutants (see Melman et al. J. Biol. Chem. 276(31):29338-29346, 2001). Particular RAP polypeptide fragments, suitable for use according to the invention, include fragments (defined from RAP N terminus amino acid to RAP C-terminus amino acid position) 1-323 (RAP); 1-319; 1-250; 1-110; 91-210; 191-323; 221-323; 1-190; 1-200; and 1-210. Preferred RAP polypeptides include fragments 1-323 (RAP); 1-319; 191-323; and 1-210. A modified RAP polypeptide having the C-terminal four amino acid sequence substituted by the sequence KDEL is also suitable. A modified RAP polypeptide in which the C-terminal-four amino acid sequence (HNEL) is deleted is also suitable. Also preferred are RAP polypeptides fragments that comprise the native sequence of RAP from amino acid 201 to 210.

Other preferred embodiments, comprise a human or mammalian RAP polypeptide in which the polypeptide comprises the native amino acid sequence of RAP over positions 282-289, 201-210, and 311-319. Mutated and N-terminus or C-terminus truncated variants of RAP which bind to the LRP receptor are disclosed in Melman et al. (J. Biol. Chem. 276(31): 29338-46, 2001) which is incorporated herein by reference in its entirety and with particularity to these RAP mutated and truncated variants. Other preferred RAP polypeptides comprise a native sequence of RAP between amino acids 85-148 and 178-248. (see Farquhar et al., Proc. Nat. Acad. Sci. USA 91:3161-3162 (1994).

Thus, many references disclose the binding sites and structure activity relationships for binding of RAP and RAP fragments to the LRP receptor. The skilled artisan can readily adapt a variety of well known techniques in the art in order to obtain RAP polypeptides that contain a LRP binding site and are suitable for use as RAP polypeptides according to the invention. The preferred fragments of RAP are soluble under physiological conditions. The N-terminus or C-terminus of these polypeptides can be shortened as desired, provided that the binding capacity for the

LRP particle remains intact. The preferred amino acid sequence of RAP corresponds to the human protein. Suitable sequences for a RAP polypeptide can also be derived from the amino acid sequences of RAP isolated from other mammals or members of the kingdom Animalia.

5 In order to generate fragments of RAP which contains the LRP binding site, isolated native protein may be converted by enzymatic and/or chemical cleavage to generate fragments of the whole protein, for example by reacting RAP with an enzyme such as papain or trypsin or a chemical such as cyanogen bromide. Proteolytically active enzymes or chemicals are preferably selected in order to release
10 the extracellular receptor region. Fragments that contain the LRP binding site, especially fragments that are soluble under physiological conditions, can then be isolated using known methods.

 Alternatively, RAP or a fragment of RAP may be expressed in a recombinant bacteria, as described, for example, in Williams et al., J. Biol. Chem. 267:9035-9040 (1992); Wurshawsky et al., J. Biol. Chem. 269:3325-3330 (1994);
15 Melman et al. J. Biol. Chem. 276(31): 29338-46 (2001).

 RAP can be in the form of acidic or basic salts, or in neutral forms. In addition, individual amino acid residues can be modified, such as by oxidation or reduction. Moreover, various substitutions, deletions, or additions can be made to the
20 amino acid or nucleic acid sequences, the net effect of which is to retain or improve upon the desired biological activity of RAP. Due to code degeneracy, for example, there may be considerable variation in nucleotide sequences encoding the same amino acid sequence.

 A RAP fragment as used herein includes, but not limited to, any
25 portion of RAP or its biologically equivalent analogs that contains a sufficient portion of the ligand to enable it to bind to LRP and to be transcytosed, transported across the blood-brain barrier; or that otherwise retains or improves upon the desired LRP mediated carrier activities of the ligand. Figure 15 shows the amino acid sequence of human RAP. Figure 16 shows the amino acid sequence of the 28 kd RAP
30 polypeptide.

 In addition to RAP, other megalin ligands may be used to facilitate the transport of active agents through transcytosis. Megalin ligands other than RAP

include, for example, include thyroglobulin (Zheng et al., *Endocrinol.*, 139:1462-1465, 1998; for exemplary sequence see e.g., GenBank Acc. No. NP_003226 and Collins et al., *J. Clin. Endocrinol. Metab.* 88 (10), 5039-5042, 2003), lipoprotein lipase (Kounnas et al., *J. Biol. Chem.*, 268:14176-14181, 1993; for exemplary

5 sequence see e.g., GenBank Acc. No. AAP35372) lactoferrin (Willnow et al., *J. Biol. Chem.*, 267: 26172-26180, 1992; for exemplary sequence see e.g., GenBank Acc. No. AAN11304 from Velliyagounder et al., *Infect. Immun.* 71 (11), 6141-6147, 2003), apolipoprotein J/clusterin (Kounnas et al., *J. Biol. Chem.*, 270:13070-13075, 1995; for exemplary sequence see e.g., GenBank Acc. No. NP_001822 and NP_976084 and Ota

10 et al., *Nat. Genet.*, *Nat. Genet.* 36 (1), 40-45 (2004); Ota et al., *Int. J. Cancer* 108 (1), 23-30, 2004), apolipoprotein B (Stefansson et al., *J. Biol. Chem.*, 270:19417-19421, 1995; for exemplary sequence see e.g., GenBank Acc. No. AAP72970), apolipoprotein E (Willnow et al., *J. Biol. Chem.*, 267: 26172-26180, 1992; for exemplary sequence see e.g., GenBank Acc. No. NP_000032 and Hirono et al., *J*

15 *Neuropsychiatry Clin Neurosci* 15 (3), 354-358, 2003), tissue type plasminogen activator (Willnow et al., *J. Biol. Chem.*, 267: 26172-26180, 1992; for exemplary sequence see e.g., GenBank Acc. No. P00750 and Pennica et al., *Nature* 301 (5897), 214-221 (1983), uPA (Moestrup et al., *J. Clin. Invest.*, 102:902-909, 1998; for exemplary sequence see e.g., GenBank Acc. No. NP_002649 and Tran et al., *Mol.*

20 *Cell. Biol.* 23 (20), 7177-7188 (2003), PAI-1 (Stefansson et al., *J. Cell. Sci.*, 108:2361-2368, 1995; for exemplary sequence see e.g., GenBank Acc. No. NP_000593 and He et al., *Biochem. Biophys. Res. Commun.* 310 (3), 878-883, 2003), vitamin D-binding protein (DBP; Nykjaer et al., *Cell* 96:507-515, 1999; for exemplary sequence see e.g., GenBank Acc. No. AAA19662 and also, Yang et al.,

25 *Gene* 54 (2-3), 285-290, 1987), vitamin A/retinol-binding protein (RBP; Christensen et al., *J. Am. Soc. Nephrol.*, 10:685-695, 1999; for exemplary sequence see e.g., GenBank Acc. No. AAA59188), β 2-microglobulin (Orlando et al., *J. Am. Soc. Nephrol.*, 9:1759-1766, 1998; AAA51811 and AAH64910), α 1-microglobulin (Orlando et al., *J. Am. Soc. Nephrol.*, 9:1759-1766, 1998; AAH41593 and

30 CAA38585), vitamin B12/cobalamin plasma carrier protein, transcobalamin (TC)-B12, PTH, insulin (Orlando et al., *J. Am. Soc. Nephrol.*, 9:1759-1766, 1998), EGF (Orlando et al., *J. Am. Soc. Nephrol.*, 9:1759-1766, 1998), prolactin (Orlando et al., *J. Am. Soc. Nephrol.*, 9:1759-1766, 1998), albumin, apo H (for exemplary sequence see e.g., GenBank Acc. No. see P02749 see also, *Gene* 108 (2), 293-298, 1991),

transthyretin (for exemplary sequence see e.g., GenBank Acc. No. see NP_000362), lysozyme (Orlando et al., J. Am. Soc. Nephrol., 9:1759-1766, 1998; see e.g., CAA00878 and EP 0222366-A), cytochrome-c (Orlando et al., J. Am. Soc. Nephrol., 9:1759-1766, 1998), α -amylase, and Ca²⁺, and aprotinin. For a detailed review of the structure, function and expression patterns of megalin those skilled in the art are referred to Christensen and Birn (Am. J. Physiol. Renal. Physiol., 280:F562-573, 2001.) It should be noted that the GenBank Acc. No. provide exemplary sequences of these proteins known to those of skill in the art. There are numerous other such sequences that also are known to those of skill that may be used in the conjugates herein either as the wild-type sequences or as modified sequences (e.g., fragments, conservative variants and the like).

Any of the above megalin ligands will be used for the delivery of active agents via transcytosis. In such embodiment, the megalin ligand is conjugated to the active agent of interest using techniques known to those of skill in the art. In preferred embodiments, it is contemplated that such ligands may be further modified to increase their binding affinity to megalin. Such modified ligands will be particularly useful delivery vehicles for transcytosis across any cell which expresses a megalin receptor. In other preferred embodiments, it is contemplated that the megalin ligands may be modified such that the ligands have a greater binding affinity for megalin than for LRP1. Such ligands will be particularly useful as vectors for the transport of proteins and small molecules across the blood-to-brain barrier. This advantage accrues from avoiding LRP1-mediated clearance of the active agents in the liver mediated through the LRP1 receptor on liver cells, thereby increasing serum residence time and, consequently, brain influx of the active agent.

V. CONJUGATES OF MEGALIN-BINDING MOIETY AND ACTIVE AGENT

Throughout the specification, Applicants refer to a megalin-binding moiety. Typically, such a moiety is a natural megalin binding ligand such as the ligands described herein above. In other embodiments, the moiety is a modified such ligand. In still further embodiments, the megalin-binding moiety may be all or part of an antibody that is immunoreactive with megalin and therefore recognizes megalin. In the present invention, the megalin-binding moiety is conjugated to an agent that is

to be delivered to a given target, e.g., to the brain. The instant specification refers to megalin ligand-active agent conjugate. It should be understood that the megalin ligand may include any of the aforementioned megalin-binding entities.

A "megalin ligand-conjugate", "ligand-polypeptide conjugate"

5 "chimeric molecule comprising a megalin ligand conjugated to an active agent" each refers to a compound comprising a ligand of megalin, or a megalin-binding fragment thereof, attached to an active agent. As used herein, the term "conjugated" means that the therapeutic agent(s) and megalin polypeptide are physically linked by, for example, by covalent chemical bonds, physical forces such van der Waals or
10 hydrophobic interactions, encapsulation, embedding, or combinations thereof. In preferred embodiments, the therapeutic agent(s) and the megalin ligand polypeptide are physically linked by covalent chemical bonds. As such, preferred chemotherapeutic agents contain a functional group such as an alcohol, acid, carbonyl, thiol or amine group to be used in the conjugation to megalin ligand or
15 fragment thereof. In preferred embodiments, the megalin ligand is RAP or a RAP polypeptide. Adriamycin is in the amine class and there is also the possibility to link through the carbonyl as well. Paclitaxel is in the alcohol class. Chemotherapeutic agents without suitable conjugation groups may be further modified to add such a group. All these compounds are contemplated in this invention. In the case of
20 multiple therapeutic agents, a combination of various conjugations can be used.

In some embodiments, a covalent chemical bond that may be either direct (no intervening atoms) or indirect (through a linker e.g., a chain of covalently linked atoms) joins the megalin ligand and the active agent. In preferred
embodiments, the megalin ligand and the active agent moiety of the conjugate are
25 directly linked by covalent bonds between an atom of the megalin ligand and an atom of the active agent. In some preferred embodiments, the megalin binding moiety is connected to the active agent moiety of the compound according to the invention by a linker that comprises a covalent bond or a peptide of virtually any amino acid sequence or any molecule or atoms capable of connecting the megalin ligand or
30 megalin binding fragment thereof to the active agent.

In some embodiments, the linker comprises a chain of atoms from 1 to about 30 atoms or longer, 2 to 5 atoms, 2 to 10 atoms, 5 to 10 atoms, or 10 to 20 atoms long. In some embodiments, the chain atoms are all carbon atoms. In some

embodiments, the chain atoms are selected from the group consisting of C, O, N, and S. Chain atoms and linkers may be selected according to their expected solubility (hydrophilicity) so as to provide a more soluble conjugate. In some embodiments, the linker provides a functional group that is subject to enzymatic attack in a lysosome.

5 In some embodiments, the linker provides a functional group which is subject to attack by an enzyme found in the target tissue or organ and which upon attack or hydrolysis severs the link between the active agent and the megalin ligand. In some embodiments, the linker provides a functional group that is subject to hydrolysis under the conditions found at the target site (e.g., low pH of a lysosome). A linker

10 may contain one or more such functional groups. In some embodiments, the length of the linker is long enough to reduce the potential for steric hindrance (when an active agent is large) between one or both of the megalin ligand binding site and the active agent active binding site.

If the linker is a covalent bond or a peptide and the active agent is a

15 polypeptide, then the entire conjugate can be a fusion protein. Such fusion proteins may be produced by recombinant genetic engineering methods known to one of ordinary skill in the art. In some embodiments, the megalin ligand portion of the conjugate is formulated to rapidly degrade to release the active compound. In other embodiments, the linker is subject to cleavage under intracellular, or more preferably,

20 lysosomal environmental conditions to release or separate the active agent portion from the megalin ligand polypeptide portion.

The conjugate can comprise one or more active agents linked to the same megalin ligand. For example, conjugation reactions may conjugate from 1 to 5, about 5, about 1 to 10, about 5 to 10, about 10 to 20, about 20 to 30, or 30 or more

25 molecules of an active agent to the megalin ligand polypeptide. These formulations can be employed as mixtures, or they may be purified into specific stoichiometric formulations. Those skilled in the art are able to determine which format and which stoichiometric ratio is preferred. Further, more than one type of active agent may be linked to the megalin ligand polypeptide where delivery of more than one type of an

30 agent to a target site or compartment is desired. A plurality of active agent species may be attached to the same megalin ligand polypeptide e.g., adriamycin-cisplatinum RAP polypeptide (or other megalin ligand) conjugates. Thus, the conjugates may consist of a range of stoichiometric ratios and incorporate more than one type of

active agent. These, too, may be separated into purified mixtures or they may be employed in aggregate.

5 The megalin ligand or fragment thereof conjugate according to the invention may be modified as desired to enhance its stability or pharmacokinetic properties (e.g., PEGylation). Suitable linkers and their functional groups for conjugating megalin ligand polypeptides and an active agent, and the synthetic chemical methods readily adaptable for preparing such, are described in U.S. Patent Application No. 60/395,762 which is assigned to the same assignee as the present application and herein incorporated by reference in its entirety.

10 The synthesis of these conjugates is efficient and convenient, producing high yields and drugs with enhanced aqueous solubility.

VI. ACTIVE AGENTS

Active agents according to the invention include agents that can affect
15 a biological process. Particularly preferred active agents for use in the compounds compositions and methods of the invention are therapeutic agents, including drugs and diagnostic agents. The term "drug" or "therapeutic agent" refers to an active agent that has a pharmacological activity or benefits health when administered in a therapeutically effective amount. Particularly preferred agents are naturally occurring
20 biological agents (e.g., enzymes, proteins, polynucleotides, antibodies, polypeptides). In some embodiments, the active agent conjugated to a megalin ligand or a megalin-binding fragment thereof (e.g., in certain preferred embodiments, a RAP or RAP polypeptide) is a molecule, as well as any binding portion or fragment thereof, that is capable of modulating a biological process in a living host. Examples of drugs or
25 therapeutic agents include substances that are used in the prevention, diagnosis, alleviation, treatment or cure of a disease or condition. It is particularly contemplated that the agent is not an agent that causes a disease. Specifically, the agent is not amyloid β protein.

A. Protein Active Agents

The active agent can be a non-protein or a protein. The active agent can be a protein or enzyme or any fragment of such that still retains some, substantially all, or all of the therapeutic or biological activity of the protein or enzyme. In some embodiments, the protein or enzyme is one that, if not expressed or produced or if substantially reduced in expression or production, would give rise to a disease, including but not limited to, lysosomal storage diseases. Preferably, the protein or enzyme is derived or obtained from a human or mouse.

In preferred embodiments of the invention, when the active agent conjugated to RAP or RAP polypeptide is a protein or enzyme, or fragment thereof possessing a biological activity of the protein or enzyme, the active agent has an amino acid sequence identical to the amino acid sequence to the corresponding portion of the human or mammalian protein or enzyme. In other embodiments, the active agent moiety of the conjugate is a protein or enzyme native to the species of the human or mammal. In other embodiments, the protein or enzyme, or fragment thereof, is substantially homologous (i.e., at least 80%, 85%, 90%, 95%, more preferably 98%, or most preferably 99% identical in amino acid sequence over a length of at least 10, 25, 50, 100, 150, or 200 amino acids, or the entire length of the active agent) to a native sequence of the corresponding human or mammal protein or enzyme.

If the compound is a protein, the compound can be an enzyme, or any fragment of an enzyme that still retains some, substantially all, or all of the activity of the enzyme. Preferably, in the treatment of lysosomal storage diseases, the enzyme is an enzyme that is found in a cell that if not expressed or produced or is substantially reduced in expression or production would give rise to a lysosomal storage disease. Preferably, the enzyme is derived or obtained from a human or mouse. Preferably, the enzyme is a lysosomal storage enzyme, such as α -L-iduronidase, iduronate-2-sulfatase, heparan N-sulfatase, α -N-acetylglucosaminidase, arylsulfatase A, galactosylceramidase, acid-alpha-glucosidase, tripeptidyl peptidase, hexosaminidase alpha, acid sphingomyelinase, β -galactosidase, or any other lysosomal storage enzyme.

In some embodiments, therefore, in the treatment of human Lysosomal Storage Diseases (LSDs), the megalin ligand -active agent conjugate comprises an active agent protein or enzyme that is deficient in the lysosomes of a subject or patient to be treated. Such enzymes, include for example, alpha-L-iduronidase, iduronate-2-sulfatase, heparan N-sulfatase, alpha-N- acetylglucosaminidase, Arylsulfatase A, Galactosylceramidase, acid-alpha-glucosidase, thioesterase, hexosaminidase A, Acid Spingomyelinase, alpha-galactosidase, or any other lysosomal storage enzyme. A table of lysosomal storage diseases and the proteins deficient therein, which are useful as active agents, follows:

10

Lysosomal Storage Disease	Protein deficiency
Mucopolysaccharidosis type I	L-Iduronidase
Mucopolysaccharidosis type II Hunter syndrome	Iduronate-2-sulfatase
Mucopolysaccharidosis type IIIA Sanfilippo syndrome	Heparan-N-sulfatase
Mucopolysaccharidosis type IIIB Sanfilippo syndrome	α -N-Acetylglucosaminidase
Mucopolysaccharidosis type IIIC Sanfilippo syndrome	AcetylCoA:N-acetyltransferase
Mucopolysaccharidosis type IID Sanfilippo syndrome	N-Acetylglucosamine 6-sulfatase
Mucopolysaccharidosis type IVA Morquio syndrome	Galactose 6-sulfatase
Mucopolysaccharidosis type IVB Morquio syndrome	β -Galactosidase
Mucopolysaccharidosis type VI	N-Acetylgalactosamine 4-sulfatase
Mucopolysaccharidosis type VII Sly syndrome	β -Glucuronidase
Mucopolysaccharidosis type IX	hyaluronoglucosaminidase
Aspartylglucosaminuria	Aspartylglucosaminidase
Cholesterol ester storage disease/Wolman disease	Acid lipase
Cystinosis	Cystine transporter
Danon disease	Lamp-2
Fabry disease	α -Galactosidase A
Farber Lipogranulomatosis/Farber disease	Acid ceramidase
Fucosidosis	α -L-Fucosidase
Galactosialidosis types I/II	Protective protein
Gaucher disease types I/II/III Gaucher	Glucocerebrosidase (β -glucosidase)

disease	
Globoid cell leukodystrophy/ Krabbe disease	Galactocerebrosidase
Glycogen storage disease II/Pompe disease	α -Glucosidase
GM1-Gangliosidosis types I/II/III	β -Galactosidase
GM2-Gangliosidosis type I/Tay Sachs disease	β -Hexosaminidase A
GM2-Gangliosidosis type II Sandhoff disease	β -Hexosaminidase A
GM2-Gangliosidosis	GM2-activator deficiency
α -Mannosidosis types I/II	α -D-Mannosidase
β -Mannosidosis	β -D-Mannosidase
Metachromatic leukodystrophy	Arylsulfatase A
Metachromatic leukodystrophy	Saposin B
Mucopolysaccharidosis type I/Sialidosis types I/II	Neuraminidase
Mucopolysaccharidosis types II /III I-cell disease	Phosphotransferase
Mucopolysaccharidosis type IIIC pseudo-Hurler polydystrophy	Phosphotransferase γ -subunit
Multiple sulfatase deficiency	Multiple sulfatases
Neuronal Ceroid Lipofuscinosis, CLN1 Batten disease	Palmitoyl protein thioesterase
Neuronal Ceroid Lipofuscinosis, CLN2 Batten disease	Tripeptidyl peptidase I
Niemann-Pick disease types A/B Niemann-Pick disease	Acid sphingomyelinase
Niemann-Pick disease type C1 Niemann-Pick disease	Cholesterol trafficking
Niemann-Pick disease type C2 Niemann-Pick disease	Cholesterol trafficking
Pycnodysostosis	Cathepsin K
Schindler disease types I/II Schindler disease	α -Galactosidase B
Sialic acid storage disease	sialic acid transporter

Thus, the lysosomal storage diseases that can be treated or prevented using the methods of the present invention include, but are not limited to, Mucopolysaccharidosis I (MPS I), MPS II, MPS IIIA, MPS IIIB, Metachromatic Leukodystrophy (MLD), Krabbe, Pompe, Ceroid Lipofuscinosis, Tay-Sachs, 5 Niemann-Pick A and B, and other lysosomal diseases.

Thus, per the above table, for each disease the conjugated agent would preferably comprise a specific active agent enzyme deficient in the disease. For instance, for methods involving MPS I, the preferred compound or enzyme is α -L- 10 iduronidase. For methods involving MPS II, the preferred compound or enzyme is

iduronate-2-sulfatase. For methods involving MPS IIIA, the preferred compound or enzyme is heparan N-sulfatase. For methods involving MPS IIIB, the preferred compound or enzyme is α -N-acetylglucosaminidase. For methods involving Metachromatic Leukodystrophy (MLD), the preferred compound or enzyme is
5 arylsulfatase A. For methods involving Krabbe, the preferred compound or enzyme is galactosylceramidase. For methods involving Pompe, the preferred compound or enzyme is acid α -glucosidase. For methods involving CLN, the preferred compound or enzyme is tripeptidyl peptidase. For methods involving Tay-Sachs, the preferred compound or enzyme is hexosaminidase alpha. For methods involving Niemann-Pick
10 A and B the preferred compound or enzyme is acid sphingomyelinase.

The megalin ligand-active agent conjugate can comprise one or more active agent moieties (e.g., 1 to 10 or 1 to 4 or 2 to 3 moieties) linked to the megalin ligand or megalin-binding fragment thereof. For example, conjugation reactions may conjugate from 1 to 4 or more molecules of alpha-L-iduronidase to a single megalin
15 ligand, such as a RAP polypeptide molecule. These formulations can be employed as mixtures, or they may be purified into specific megalin ligand polypeptide-agent stoichiometric formulations. Those skilled in the art are able to determine which format and which stoichiometric ratio is preferred. Further, one or more different active agents may be linked to any given molecule of a megalin ligand or a megalin-
20 binding fragment of a megalin ligand to facilitate a more complete degradation of the stored substrates. These megalin ligand conjugated agents may consist of a range of stoichiometric ratios. These, too, may be separated into purified mixtures or they may be employed in aggregate. It may be the order of megalin-binding moiety and the LSD in the fusion is important for the ability of megalin binding moiety to bind to
25 megalin. Therefore, in preferred embodiments, the megalin-binding moiety is located N-terminally to the LSD enzyme coding sequence. In specific embodiments, it is contemplated that the conjugates of the invention comprise a RAP encoding sequence located N-terminally to the LSD enzyme coding sequence.

The megalin ligand conjugated active agents can enter or be
30 transported into or end up residing in the lysosomes of a cell within or without the CNS. The rate of passage of the conjugated agent can be modulated by any compound or protein that can modulate megalin binding activity. In preferred embodiments, the megalin binding affinity of the conjugate is higher than the LRP1

binding affinity. The cell can be from any tissue or organ system affected by the lysosomal storage disease. The cell can be, for instance, an endothelial, epithelial, muscle, heart, bone, lung, fat, kidney, or liver cell. In some embodiments, the cell is preferably a cell found within the BBB. In some embodiments, the cell is a neuron or
5 a brain cell. In other embodiments, the cell is a cell of the periphery or one that is not isolated from the general circulation by an endothelium such as that of the BBB.

B. Drug Active Agents

Generally, the drug active agent may be of any size. Preferred drugs
10 are small organic molecules that are capable of binding to the target of interest. A drug moiety of the conjugate, when a small molecule, generally has a molecular weight of at least about 50 D, usually at least about 100 D, where the molecular weight may be as high as 500 D or higher, but will usually not exceed about 2000 D.

The drug moiety is capable of interacting with a target in the host into
15 which the conjugate is administered during practice of the subject methods. The target may be a number of different types of naturally occurring structures, where targets of interest include both intracellular and extracellular targets, where such targets may be proteins, phospholipids, nucleic acids and the like, where proteins are of particular interest. Specific proteinaceous targets of interest include, without
20 limitation, enzymes, e.g., kinases, phosphatases, reductases, cyclooxygenases, proteases and the like, targets comprising domains involved in protein-protein interactions, such as the SH2, SH3, PTB and PDZ domains, structural proteins, e.g., actin, tubulin, etc., membrane receptors, immunoglobulins, e.g., IgE, cell adhesion receptors, such as integrins, etc., ion channels, transmembrane pumps, transcription
25 factors, signaling proteins, and the like.

In some embodiments, the active agent or drug has a hydroxyl or an amino group for reacting with the isocyanate reagent or the active agent is chemically modified to introduce a hydroxyl or an amino group for reacting with the isocyanate reagent.

30 In some embodiments, the active agent or drug comprises a region that may be modified and/or participate in covalent linkage, preferably, without loss of the desired biological activity of the active agent. The drug moieties often comprise

cyclical carbon or heterocyclic structures and/or aromatic or polyaromatic structures substituted with one or more of the above functional groups. Also of interest as drug moieties are structures found among biomolecules, proteins, enzymes, polysaccharides, and polynucleic acids, including peptides, saccharides, fatty acids, steroids, purines, pyrimidines, derivatives, structural analogs or combinations thereof.

Suitable active agents include, but are not limited to, psychopharmacological agents, such as (1) central nervous system depressants, e.g., general anesthetics (barbiturates, benzodiazepines, steroids, cyclohexanone derivatives, and miscellaneous agents), sedative-hypnotics (benzodiazepines, barbiturates, piperidinediones and triones, quinazoline derivatives, carbamates, aldehydes and derivatives, amides, acyclic ureides, benzazepines and related drugs, phenothiazines, etc.), central voluntary muscle tone modifying drugs (anticonvulsants, such as hydantoins, barbiturates, oxazolidinediones, succinimides, acylureides, glutarimides, benzodiazepines, secondary and tertiary alcohols, dibenzazepine derivatives, valproic acid and derivatives, GABA analogs, etc.), analgesics (morphine and derivatives, oripavine derivatives, morphinan derivatives, phenylpiperidines, 2,6-methane-3-benzazocaine derivatives, diphenylpropylamines and isosteres, salicylates, p-aminophenol derivatives, 5-pyrazolone derivatives, arylacetic acid derivatives, fenamates and isosteres, etc.) and antiemetics (anticholinergics, antihistamines, antidopaminergics, etc.), (2) central nervous system stimulants, e.g., analeptics (respiratory stimulants, convulsant stimulants, psychomotor stimulants), narcotic antagonists (morphine derivatives, oripavine derivatives, 2,6-methane-3-benzoxacine derivatives, morphinan derivatives) nootropics, (3) psychopharmacologicals, e.g., anxiolytic sedatives (benzodiazepines, propanediol carbamates) antipsychotics (phenothiazine derivatives, thioxanthine derivatives, other tricyclic compounds, butyrophenone derivatives and isosteres, diphenylbutylamine derivatives, substituted benzamides, arylpiperazine derivatives, indole derivatives, etc.), antidepressants (tricyclic compounds, MAO inhibitors, etc.), (4) respiratory tract drugs, e.g., central antitussives (opium alkaloids and their derivatives); pharmacodynamic agents, such as (1) peripheral nervous system drugs, e.g., local anesthetics (ester derivatives, amide derivatives), (2) drugs acting at synaptic or neuroeffector junctional sites, e.g., cholinergic agents, cholinergic blocking agents, neuromuscular blocking agents, adrenergic agents, antiadrenergic agents, (3) smooth muscle active drugs, e.g.,

spasmolytics (anticholinergics, musculotropic spasmolytics), vasodilators, smooth muscle stimulants, (4) histamines and antihistamines, e.g., histamine and derivative thereof (betazole), antihistamines (H1 -antagonists, H2 -antagonists), histamine metabolism drugs, (5) cardiovascular drugs, e.g., cardiotonics (plant extracts, butenolides, pentadienolids, alkaloids from erythrophleum species, ionophores, adrenoceptor stimulants, etc), antiarrhythmic drugs, antihypertensive agents, antilipidemic agents (clofibric acid derivatives, nicotinic acid derivatives, hormones and analogs, antibiotics, salicylic acid and derivatives), antivaricose drugs, hemostyptics, (6) blood and hemopoietic system drugs, e.g., antianemia drugs, blood coagulation drugs (hemostatics, anticoagulants, antithrombotics, thrombolytics, blood proteins and their fractions), (7) gastrointestinal tract drugs, e.g., digestants (stomachics, cholereitics), antiulcer drugs, antidiarrheal agents, (8) locally acting drugs; chemotherapeutic agents, such as (1) anti-infective agents, e.g., ectoparasitocides (chlorinated hydrocarbons, pyrethins, sulfurated compounds), antihelminthics, antiprotozoal agents, antimalarial agents, antiamebic agents, antileishmanial drugs, antitrichomonal agents, antitrypanosomal agents, sulfonamides, antimycobacterial drugs, antiviral chemotherapeutics, etc., and (2) cytostatics, i.e., antineoplastic agents or cytotoxic drugs, such as alkylating agents, e.g., Mechlorethamine hydrochloride (Nitrogen Mustard, Mustargen, HN2), Cyclophosphamide (Cytovan, Endoxana), Ifosfamide (IFEX), Chlorambucil (Leukeran), Melphalan (Phenylalanine Mustard, L-sarcolysin, Alkeran, L-PAM), Busulfan (Myleran), Thiotepa (Triethylenethiophosphoramide), Carmustine (BiCNU, BCNU), Lomustine (CeeNU, CCNU), Streptozocin (Zanosar) and the like; plant alkaloids, e.g., Vincristine (Oncovin), Vinblastine (Velban, Velbe), Paclitaxel (Taxol), and the like; antimetabolites, e.g., Methotrexate (MTX), Mercaptopurine (Purinethol, 6-MP), Thioguanine (6-TG), Fluorouracil (5-FU), Cytarabine (Cytosar-U, Ara-C), Azacitidine (Mylosar, 5-AZA) and the like; antibiotics, e.g., Dactinomycin (Actinomycin D, Cosmegen), Doxorubicin (Adriamycin), Daunorubicin (daunomycin, Cerubidine), Idarubicin (Idamycin), Bleomycin (Blenoxane), Picamycin (Mithramycin, Mithracin), Mitomycin (Mutamycin) and the like, and other anticellular proliferative agents, e.g., Hydroxyurea (Hydrea), Procarbazine (Mutalane), Dacarbazine (DTIC-Dome), Cisplatin (Platinol) Carboplatin (Paraplatin), Asparaginase (Elspar) Etoposide (VePesid, VP-16-213), Amsacrine (AMSA, m-AMSA), Mitotane (Lysodren), Mitoxantrone (Novatrone), and the like. Preferred

chemotherapeutic agents are those, which in the free form, demonstrate unacceptable systemic toxicity at desired doses. The general systemic toxicity associated with therapeutic levels of such agents may be reduced by their linkage to RAP or a RAP polypeptide or other megalin ligand. Particularly preferred are cardiotoxic

- 5 compounds that are useful therapeutics but are dose limited by cardiotoxicity. A classic example is adriamycin (also known as doxorubicin) and its analogs, such as daunorubicin. Linking RAP or a RAP polypeptide or another megalin ligand or a megalin-binding fragment of such a ligand to such drugs may prevent accumulation of the active agent at the heart and associated cardiotoxicity.

- 10 Suitable active agents include, but are not limited to: Antibiotics, such as: aminoglycosides, e.g., amikacin, apramycin, arbekacin, bambarmycins, butirosin, dibekacin, dihydrostreptomycin, fortimicin, gentamicin, isepamicin, kanamycin, micronomycin, neomycin, netilmicin, paromycin, ribostamycin, sisomicin, spectinomycin, streptomycin, tobramycin, trospectomycin; amphenicols, e.g.,
 15 azidamfenicol, chloramphenicol, florfenicol, and theimaphenicol; ansamycins, e.g., rifamide, rifampin, rifamycin, rifapentine, rifaximin; beta.-lactams, e.g., carbacephems, carbapenems, cephalosporins, cephamycins, monobactams, oxaphems, penicillins; lincosamides, e.g., clindamycin, lincomycin; macrolides, e.g., clarithromycin, dirithromycin, erythromycin, etc.; polypeptides, e.g., amphotericin,
 20 bacitracin, capreomycin, etc.; tetracyclines, e.g., apicycline, chlortetracycline, clomocycline, etc.; synthetic antibacterial agents, such as 2,4-diaminopyrimidines, nitrofurans, quinolones and analogs thereof, sulfonamides, sulfones;

- Suitable active agents include, but are not limited to: Antifungal agents, such as: polyenes, e.g., amphotericin B, candicidin, dermostatin, filipin,
 25 fungichromin, hachimycin, hamycin, lucensomycin, mepartricin, natamycin, nystatin, pecilocin, perimycin; synthetic antifungals, such as allylamines, e.g., butenafine, naftifine, terbinafine; imidazoles, e.g., bifonazole, butoconazole, chlordanol, chlormidazole, etc., thiocarbamates, e.g., tolclate, triazoles, e.g., fluconazole, itraconazole, terconazole;

- 30 Suitable active agents include, but are not limited to: Anthelmintics, such as: arecoline, aspidin, aspidinol, dichlorophene, embelin, kosin, naphthalene, niclosamide, pelletierine, quinacrine, alantolactone, amocarzone, amoscanate,

ascaridole, buphenium, bitoscanate, carbon tetrachloride, carvacrol, cyclobendazole, diethylcarbamazine, etc.;

Suitable active agents include, but are not limited to: Antimalarials, such as: acedapsone, amodiaquin, arteether, artemether, artemisinin, artesunate, 5 atovaquone, bebeerine, berberine, chirata, chlorguanide, chloroquine, chlorproguanil, cinchona, cinchonidine, cinchonine, cycloguanil, gentiopicrin, halofantrine, hydroxychloroquine, mefloquine hydrochloride, 3-methylarsacetin, pamaquine, plasmocid, primaquine, pyrimethamine, quinacrine, quinidine, quinine, quinic acid, quinoline, dibasic sodium arsenate;

10 Suitable active agents include, but are not limited to: Antiprotozoan agents, such as: acranil, tinidazole, ipronidazole, ethylstibamine, pentamidine, acetarsone, aminitroazole, anisomycin, nifuratel, tinidazole, benzidazole, suramin, and the like.

Suitable drugs for use as active agents are also listed in: Goodman and 15 Gilman's, The Pharmacological Basis of Therapeutics (9th Ed) (Goodman et al. eds) (McGraw-Hill) (1996); and 1999 Physician's Desk Reference (1998).

Suitable active agents include, but are not limited to: antineoplastic agents, as disclosed in U.S. Pat. Nos. 5,880,161, 5,877,206, 5,786,344, 5,760,041, 5,753,668, 5,698,529, 5,684,004, 5,665,715, 5,654,484, 5,624,924, 5,618,813, 20 5,610,292, 5,597,831, 5,530,026, 5,525,633, 5,525,606, 5,512,678, 5,508,277, 5,463,181, 5,409,893, 5,358,952, 5,318,965, 5,223,503, 5,214,068, 5,196,424, 5,109,024, 5,106,996, 5,101,072, 5,077,404, 5,071,848, 5,066,493, 5,019,390, 4,996,229, 4,996,206, 4,970,318, 4,968,800, 4,962,114, 4,927,828, 4,892,887, 4,889,859, 4,886,790, 4,882,334, 4,882,333, 4,871,746, 4,863,955, 4,849,563, 25 4,845,216, 4,833,145, 4,824,955, 4,785,085, 4,684,747, 4,618,685, 4,611,066, 4,550,187, 4,550,186, 4,544,501, 4,541,956, 4,532,327, 4,490,540, 4,399,283, 4,391,982, 4,383,994, 4,294,763, 4,283,394, 4,246,411, 4,214,089, 4,150,231, 4,147,798, 4,056,673, 4,029,661, 4,012,448;

psychopharmacological/psychotropic agents, as disclosed in U.S. Pat. 30 Nos. 5,192,799, 5,036,070, 4,778,800, 4,753,951, 4,590,180, 4,690,930, 4,645,773, 4,427,694, 4,424,202, 4,440,781, 5,686,482, 5,478,828, 5,461,062, 5,387,593, 5,387,586, 5,256,664, 5,192,799, 5,120,733, 5,036,070, 4,977,167, 4,904,663,

4,788,188, 4,778,800, 4,753,951, 4,690,930, 4,645,773, 4,631,285, 4,617,314,
4,613,600, 4,590,180, 4,560,684, 4,548,938, 4,529,727, 4,459,306, 4,443,451,
4,440,781, 4,427,694, 4,424,202, 4,397,853, 4,358,451, 4,324,787, 4,314,081,
4,313,896, 4,294,828, 4,277,476, 4,267,328, 4,264,499, 4,231,930, 4,194,009,
5 4,188,388, 4,148,796, 4,128,717, 4,062,858, 4,031,226, 4,020,072, 4,018,895,
4,018,779, 4,013,672, 3,994,898, 3,968,125, 3,939,152, 3,928,356, 3,880,834,
3,668,210;

cardiovascular agents, as disclosed in U.S. Pat. Nos. 4,966,967,
5,661,129, 5,552,411, 5,332,737, 5,389,675, 5,198,449, 5,079,247, 4,966,967,
10 4,874,760, 4,954,526, 5,051,423, 4,888,335, 4,853,391, 4,906,634, 4,775,757,
4,727,072, 4,542,160, 4,522,949, 4,524,151, 4,525,479, 4,474,804, 4,520,026,
4,520,026, 5,869,478, 5,859,239, 5,837,702, 5,807,889, 5,731,322, 5,726,171,
5,723,457, 5,705,523, 5,696,111, 5,691,332, 5,679,672, 5,661,129, 5,654,294,
5,646,276, 5,637,586, 5,631,251, 5,612,370, 5,612,323, 5,574,037, 5,563,170,
15 5,552,411, 5,552,397, 5,547,966, 5,482,925, 5,457,118, 5,414,017, 5,414,013,
5,401,758, 5,393,771, 5,362,902, 5,332,737, 5,310,731, 5,260,444, 5,223,516,
5,217,958, 5,208,245, 5,202,330, 5,198,449, 5,189,036, 5,185,362, 5,140,031,
5,128,349, 5,116,861, 5,079,247, 5,070,099, 5,061,813, 5,055,466, 5,051,423,
5,036,065, 5,026,712, 5,011,931, 5,006,542, 4,981,843, 4,977,144, 4,971,984,
20 4,966,967, 4,959,383, 4,954,526, 4,952,692, 4,939,137, 4,906,634, 4,889,866,
4,888,335, 4,883,872, 4,883,811, 4,847,379, 4,835,157, 4,824,831, 4,780,538,
4,775,757, 4,774,239, 4,771,047, 4,769,371, 4,767,756, 4,762,837, 4,753,946,
4,752,616, 4,749,715, 4,738,978, 4,735,962, 4,734,426, 4,734,425, 4,734,424,
4,730,052, 4,727,072, 4,721,796, 4,707,550, 4,704,382, 4,703,120, 4,681,970,
25 4,681,882, 4,670,560, 4,670,453, 4,668,787, 4,663,337, 4,663,336, 4,661,506,
4,656,267, 4,656,185, 4,654,357, 4,654,356, 4,654,355, 4,654,335, 4,652,578,
4,652,576, 4,650,874, 4,650,797, 4,649,139, 4,647,585, 4,647,573, 4,647,565,
4,647,561, 4,645,836, 4,639,461, 4,638,012, 4,638,011, 4,632,931, 4,631,283,
4,628,095, 4,626,548, 4,614,825, 4,611,007, 4,611,006, 4,611,005, 4,609,671,
30 4,608,386, 4,607,049, 4,607,048, 4,595,692, 4,593,042, 4,593,029, 4,591,603,
4,588,743, 4,588,742, 4,588,741, 4,582,854, 4,575,512, 4,568,762, 4,560,698,
4,556,739, 4,556,675, 4,555,571, 4,555,570, 4,555,523, 4,550,120, 4,542,160,
4,542,157, 4,542,156, 4,542,155, 4,542,151, 4,537,981, 4,537,904, 4,536,514,

4,536,513, 4,533,673, 4,526,901, 4,526,900, 4,525,479, 4,524,151, 4,522,949,
4,521,539, 4,520,026, 4,517,188, 4,482,562, 4,474,804, 4,474,803, 4,472,411,
4,466,979, 4,463,015, 4,456,617, 4,456,616, 4,456,615, 4,418,076, 4,416,896,
4,252,815, 4,220,594, 4,190,587, 4,177,280, 4,164,586, 4,151,297, 4,145,443,
5 4,143,054, 4,123,550, 4,083,968, 4,076,834, 4,064,259, 4,064,258, 4,064,257,
4,058,620, 4,001,421, 3,993,639, 3,991,057, 3,982,010, 3,980,652, 3,968,117,
3,959,296, 3,951,950, 3,933,834, 3,925,369, 3,923,818, 3,898,210, 3,897,442,
3,897,441, 3,886,157, 3,883,540, 3,873,715, 3,867,383, 3,873,715, 3,867,383,
3,691,216, 3,624,126;

10 antimicrobial agents as disclosed in U.S. Pat. Nos. 5,902,594,
5,874,476, 5,874,436, 5,859,027, 5,856,320, 5,854,242, 5,811,091, 5,786,350,
5,783,177, 5,773,469, 5,762,919, 5,753,715, 5,741,526, 5,709,870, 5,707,990,
5,696,117, 5,684,042, 5,683,709, 5,656,591, 5,643,971, 5,643,950, 5,610,196,
5,608,056, 5,604,262, 5,595,742, 5,576,341, 5,554,373, 5,541,233, 5,534,546,
15 5,534,508, 5,514,715, 5,508,417, 5,464,832, 5,428,073, 5,428,016, 5,424,396,
5,399,553, 5,391,544, 5,385,902, 5,359,066, 5,356,803, 5,354,862, 5,346,913,
5,302,592, 5,288,693, 5,266,567, 5,254,685, 5,252,745, 5,209,930, 5,196,441,
5,190,961, 5,175,160, 5,157,051, 5,096,700, 5,093,342, 5,089,251, 5,073,570,
5,061,702, 5,037,809, 5,036,077, 5,010,109, 4,970,226, 4,916,156, 4,888,434,
20 4,870,093, 4,855,318, 4,784,991, 4,746,504, 4,686,221, 4,599,228, 4,552,882,
4,492,700, 4,489,098, 4,489,085, 4,487,776, 4,479,953, 4,477,448, 4,474,807,
4,470,994, 4,370,484, 4,337,199, 4,311,709, 4,308,283, 4,304,910, 4,260,634,
4,233,311, 4,215,131, 4,166,122, 4,141,981, 4,130,664, 4,089,977, 4,089,900,
4,069,341, 4,055,655, 4,049,665, 4,044,139, 4,002,775, 3,991,201, 3,966,968,
25 3,954,868, 3,936,393, 3,917,476, 3,915,889, 3,867,548, 3,865,748, 3,867,548,
3,865,748, 3,783,160, 3,764,676, 3,764,677;

anti-inflammatory agents as disclosed in U.S. Pat. Nos. 5,872,109,
5,837,735, 5,827,837, 5,821,250, 5,814,648, 5,780,026, 5,776,946, 5,760,002,
5,750,543, 5,741,798, 5,739,279, 5,733,939, 5,723,481, 5,716,967, 5,688,949,
30 5,686,488, 5,686,471, 5,686,434, 5,684,204, 5,684,041, 5,684,031, 5,684,002,
5,677,318, 5,674,891, 5,672,620, 5,665,752, 5,656,661, 5,635,516, 5,631,283,
5,622,948, 5,618,835, 5,607,959, 5,593,980, 5,593,960, 5,580,888, 5,552,424,
5,552,422, 5,516,764, 5,510,361, 5,508,026, 5,500,417, 5,498,405, 5,494,927,

5,476,876, 5,472,973, 5,470,885, 5,470,842, 5,464,856, 5,464,849, 5,462,952,
5,459,151, 5,451,686, 5,444,043, 5,436,265, 5,432,181, RE034918, 5,393,756,
5,380,738, 5,376,670, 5,360,811, 5,354,768, 5,348,957, 5,347,029, 5,340,815,
5,338,753, 5,324,648, 5,319,099, 5,318,971, 5,312,821, 5,302,597, 5,298,633,
5 5,298,522, 5,298,498, 5,290,800, 5,290,788, 5,284,949, 5,280,045, 5,270,319,
5,266,562, 5,256,680, 5,250,700, 5,250,552, 5,248,682, 5,244,917, 5,240,929,
5,234,939, 5,234,937, 5,232,939, 5,225,571, 5,225,418, 5,220,025, 5,212,189,
5,212,172, 5,208,250, 5,204,365, 5,202,350, 5,196,431, 5,191,084, 5,187,175,
5,185,326, 5,183,906, 5,177,079, 5,171,864, 5,169,963, 5,155,122, 5,143,929,
10 5,143,928, 5,143,927, 5,124,455, 5,124,347, 5,114,958, 5,112,846, 5,104,656,
5,098,613, 5,095,037, 5,095,019, 5,086,064, 5,081,261, 5,081,147, 5,081,126,
5,075,330, 5,066,668, 5,059,602, 5,043,457, 5,037,835, 5,037,811, 5,036,088,
5,013,850, 5,013,751, 5,013,736, 5,006,542, 4,992,448, 4,992,447, 4,988,733,
4,988,728, 4,981,865, 4,962,119, 4,959,378, 4,954,519, 4,945,099, 4,942,236,
15 4,931,457, 4,927,835, 4,912,248, 4,910,192, 4,904,786, 4,904,685, 4,904,674,
4,904,671, 4,897,397, 4,895,953, 4,891,370, 4,870,210, 4,859,686, 4,857,644,
4,853,392, 4,851,412, 4,847,303, 4,847,290, 4,845,242, 4,835,166, 4,826,990,
4,803,216, 4,801,598, 4,791,129, 4,788,205, 4,778,818, 4,775,679, 4,772,703,
4,767,776, 4,764,525, 4,760,051, 4,748,153, 4,725,616, 4,721,712, 4,713,393,
20 4,708,966, 4,695,571, 4,686,235, 4,686,224, 4,680,298, 4,678,802, 4,652,564,
4,644,005, 4,632,923, 4,629,793, 4,614,741, 4,599,360, 4,596,828, 4,595,694,
4,595,686, 4,594,357, 4,585,755, 4,579,866, 4,578,390, 4,569,942, 4,567,201,
4,563,476, 4,559,348, 4,558,067, 4,556,672, 4,556,669, 4,539,326, 4,537,903,
4,536,503, 4,518,608, 4,514,415, 4,512,990, 4,501,755, 4,495,197, 4,493,839,
25 4,465,687, 4,440,779, 4,440,763, 4,435,420, 4,412,995, 4,400,534, 4,355,034,
4,335,141, 4,322,420, 4,275,064, 4,244,963, 4,235,908, 4,234,593, 4,226,887,
4,201,778, 4,181,720, 4,173,650, 4,173,634, 4,145,444, 4,128,664, 4,125,612,
4,124,726, 4,124,707, 4,117,135, 4,027,031, 4,024,284, 4,021,553, 4,021,550,
4,018,923, 4,012,527, 4,011,326, 3,998,970, 3,998,954, 3,993,763, 3,991,212,
30 3,984,405, 3,978,227, 3,978,219, 3,978,202, 3,975,543, 3,968,224, 3,959,368,
3,949,082, 3,949,081, 3,947,475, 3,936,450, 3,934,018, 3,930,005, 3,857,955,
3,856,962, 3,821,377, 3,821,401, 3,789,121, 3,789,123, 3,726,978, 3,694,471,
3,691,214, 3,678,169, 3,624,216;

immunosuppressive agents, as disclosed in U.S. Pat. Nos. 4,450,159, 4,450,159, 5,905,085, 5,883,119, 5,880,280, 5,877,184, 5,874,594, 5,843,452, 5,817,672, 5,817,661, 5,817,660, 5,801,193, 5,776,974, 5,763,478, 5,739,169, 5,723,466, 5,719,176, 5,696,156, 5,695,753, 5,693,648, 5,693,645, 5,691,346, 5 5,686,469, 5,686,424, 5,679,705, 5,679,640, 5,670,504, 5,665,774, 5,665,772, 5,648,376, 5,639,455, 5,633,277, 5,624,930, 5,622,970, 5,605,903, 5,604,229, 5,574,041, 5,565,560, 5,550,233, 5,545,734, 5,540,931, 5,532,248, 5,527,820, 5,516,797, 5,514,688, 5,512,687, 5,506,233, 5,506,228, 5,494,895, 5,484,788, 5,470,857, 5,464,615, 5,432,183, 5,431,896, 5,385,918, 5,349,061, 5,344,925, 10 5,330,993, 5,308,837, 5,290,783, 5,290,772, 5,284,877, 5,284,840, 5,273,979, 5,262,533, 5,260,300, 5,252,732, 5,250,678, 5,247,076, 5,244,896, 5,238,689, 5,219,884, 5,208,241, 5,208,228, 5,202,332, 5,192,773, 5,189,042, 5,169,851, 5,162,334, 5,151,413, 5,149,701, 5,147,877, 5,143,918, 5,138,051, 5,093,338, 5,091,389, 5,068,323, 5,068,247, 5,064,835, 5,061,728, 5,055,290, 4,981,792, 15 4,810,692, 4,410,696, 4,346,096, 4,342,769, 4,317,825, 4,256,766, 4,180,588, 4,000,275, 3,759,921;

immunomodulatory agents, as disclosed in U.S. Pat. Nos. 4,446,128, 4,524,147, 4,720,484, 4,722,899, 4,748,018, 4,877,619, 4,998,931, 5,049,387, 5,118,509, 5,152,980, 5,256,416, 5,468,729, 5,583,139, 5,604,234, 5,612,060, 20 5,612,350, 5,658,564, 5,672,605, 5,681,571, 5,708,002, 5,723,718, 5,736,143, 5,744,495, 5,753,687, 5,770,201, 5,869,057, 5,891,653, 5,939,455, 5,948,407, 6,006,752, 6,024,957, 6,030,624, 6,037,372, 6,037,373, 6,043,247, 6,060,049, 6,087,096, 6,096,315, 6,099,838, 6,103,235, 6,124,495, 6,153,203, 6,169,087, 6,255,278, 6,262,044, 6,290,950, 6,306,651, 6,322,796, 6,329,153, 6,344,476, 25 6,352,698, 6,365,163, 6,379,668, 6,391,303, 6,395,767, 6,403,555, 6,410,556, 6,412,492, 6,468,537, 6,489,330, 6,521,232, 6,525,035, 6,525,242, 6,558,663, 6,572,860;

analgesic agents, as disclosed in U.S. Pat. Nos. 5,292,736, 5,688,825, 5,554,789, 5,455,230, 5,292,736, 5,298,522, 5,216,165, 5,438,064, 5,204,365, 30 5,017,578, 4,906,655, 4,906,655, 4,994,450, 4,749,792, 4,980,365, 4,794,110, 4,670,541, 4,737,493, 4,622,326, 4,536,512, 4,719,231, 4,533,671, 4,552,866, 4,539,312, 4,569,942, 4,681,879, 4,511,724, 4,556,672, 4,721,712, 4,474,806, 4,595,686, 4,440,779, 4,434,175, 4,608,374, 4,395,402, 4,400,534, 4,374,139,

- 4,361,583, 4,252,816, 4,251,530, 5,874,459, 5,688,825, 5,554,789, 5,455,230,
5,438,064, 5,298,522, 5,216,165, 5,204,365, 5,030,639, 5,017,578, 5,008,264,
4,994,450, 4,980,365, 4,906,655, 4,847,290, 4,844,907, 4,794,110, 4,791,129,
4,774,256, 4,749,792, 4,737,493, 4,721,712, 4,719,231, 4,681,879, 4,670,541,
5 4,667,039, 4,658,037, 4,634,708, 4,623,648, 4,622,326, 4,608,374, 4,595,686,
4,594,188, 4,569,942, 4,556,672, 4,552,866, 4,539,312, 4,536,512, 4,533,671,
4,511,724, 4,440,779, 4,434,175, 4,400,534, 4,395,402, 4,391,827, 4,374,139,
4,361,583, 4,322,420, 4,306,097, 4,252,816, 4,251,530, 4,244,955, 4,232,018,
4,209,520, 4,164,514, 4,147,872, 4,133,819, 4,124,713, 4,117,012, 4,064,272,
10 4,022,836, 3,966,944;
- cholinergic agents, as disclosed in U.S. Pat. Nos. 5,219,872, 5,219,873,
5,073,560, 5,073,560, 5,346,911, 5,424,301, 5,073,560, 5,219,872, 4,900,748,
4,786,648, 4,798,841, 4,782,071, 4,710,508, 5,482,938, 5,464,842, 5,378,723,
5,346,911, 5,318,978, 5,219,873, 5,219,872, 5,084,281, 5,073,560, 5,002,955,
15 4,988,710, 4,900,748, 4,798,841, 4,786,648, 4,782,071, 4,745,123, 4,710,508;
- adrenergic agents, as disclosed in U.S. Pat. Nos. 5,091,528, 5,091,528,
4,835,157, 5,708,015, 5,594,027, 5,580,892, 5,576,332, 5,510,376, 5,482,961,
5,334,601, 5,202,347, 5,135,926, 5,116,867, 5,091,528, 5,017,618, 4,835,157,
4,829,086, 4,579,867, 4,568,679, 4,469,690, 4,395,559, 4,381,309, 4,363,808,
20 4,343,800, 4,329,289, 4,314,943, 4,311,708, 4,304,721, 4,296,117, 4,285,873,
4,281,189, 4,278,608, 4,247,710, 4,145,550, 4,145,425, 4,139,535, 4,082,843,
4,011,321, 4,001,421, 3,982,010, 3,940,407, 3,852,468, 3,832,470;
- antihistamine agents, as disclosed in U.S. Pat. Nos. 5,874,479,
5,863,938, 5,856,364, 5,770,612, 5,702,688, 5,674,912, 5,663,208, 5,658,957,
25 5,652,274, 5,648,380, 5,646,190, 5,641,814, 5,633,285, 5,614,561, 5,602,183,
4,923,892, 4,782,058, 4,393,210, 4,180,583, 3,965,257, 3,946,022, 3,931,197;
- steroidal agents, as disclosed in U.S. Pat. Nos. 5,863,538, 5,855,907,
5,855,866, 5,780,592, 5,776,427, 5,651,987, 5,346,887, 5,256,408, 5,252,319,
5,209,926, 4,996,335, 4,927,807, 4,910,192, 4,710,495, 4,049,805, 4,004,005,
30 3,670,079, 3,608,076, 5,892,028, 5,888,995, 5,883,087, 5,880,115, 5,869,475,
5,866,558, 5,861,390, 5,861,388, 5,854,235, 5,837,698, 5,834,452, 5,830,886,
5,792,758, 5,792,757, 5,763,361, 5,744,462, 5,741,787, 5,741,786, 5,733,899,

5,731,345, 5,723,638, 5,721,226, 5,712,264, 5,712,263, 5,710,144, 5,707,984,
 5,705,494, 5,700,793, 5,698,720, 5,698,545, 5,696,106, 5,677,293, 5,674,861,
 5,661,141, 5,656,621, 5,646,136, 5,637,691, 5,616,574, 5,614,514, 5,604,215,
 5,604,213, 5,599,807, 5,585,482, 5,565,588, 5,563,259, 5,563,131, 5,561,124,
 5 5,556,845, 5,547,949, 5,536,714, 5,527,806, 5,506,354, 5,506,221, 5,494,907,
 5,491,136, 5,478,956, 5,426,179, 5,422,262, 5,391,776, 5,382,661, 5,380,841,
 5,380,840, 5,380,839, 5,373,095, 5,371,078, 5,352,809, 5,344,827, 5,344,826,
 5,338,837, 5,336,686, 5,292,906, 5,292,878, 5,281,587, 5,272,140, 5,244,886,
 5,236,912, 5,232,915, 5,219,879, 5,218,109, 5,215,972, 5,212,166, 5,206,415,
 10 5,194,602, 5,166,201, 5,166,055, 5,126,488, 5,116,829, 5,108,996, 5,099,037,
 5,096,892, 5,093,502, 5,086,047, 5,084,450, 5,082,835, 5,081,114, 5,053,404,
 5,041,433, 5,041,432, 5,034,548, 5,032,586, 5,026,882, 4,996,335, 4,975,537,
 4,970,205, 4,954,446, 4,950,428, 4,946,834, 4,937,237, 4,921,846, 4,920,099,
 4,910,226, 4,900,725, 4,892,867, 4,888,336, 4,885,280, 4,882,322, 4,882,319,
 15 4,882,315, 4,874,855, 4,868,167, 4,865,767, 4,861,875, 4,861,765, 4,861,763,
 4,847,014, 4,774,236, 4,753,932, 4,711,856, 4,710,495, 4,701,450, 4,701,449,
 4,689,410, 4,680,290, 4,670,551, 4,664,850, 4,659,516, 4,647,410, 4,634,695,
 4,634,693, 4,588,530, 4,567,000, 4,560,557, 4,558,041, 4,552,871, 4,552,868,
 4,541,956, 4,519,946, 4,515,787, 4,512,986, 4,502,989, 4,495,102; the disclosures of
 20 all the above of which are herein incorporated by reference.

The drug moiety of the conjugate may be the whole drug or a binding
 fragment or portion thereof that retains its affinity and specificity for the target of
 interest while having a linkage site for covalent bonding to the vector protein ligand
 or linker. The conjugates of such drugs may be used for the same disorders, diseases,
 25 and indications as the drugs themselves.

C. Preferred Cancer Chemotherapeutic Active Agents

Preferred cancer chemotherapeutic agents for use in the megalin ligand
 based conjugates of the invention include all drugs which may be useful for treating
 brain tumors or other neoplasia in or around the brain, either in the free form, or, if
 30 not so useful for such tumors in the free form, then useful when linked to the megalin
 ligand or megalin binding fragment thereof. Such chemotherapeutic agents are
 preferably cytotoxic chemotherapeutic agents including but not limited to adriamycin
 (also known as doxorubicin), cisplatin, paclitaxel, analogs thereof, and other

chemotherapeutic agents demonstrate activity against tumours ex vivo and in vivo. Such chemotherapeutic agents also include alkylating agents, antimetabolites, natural products (such as vinca alkaloids, epidophyllotoxins, antibiotics, enzymes and biological response modifiers), topoisomerase inhibitors, microtubule inhibitors, 5 spindle poisons, hormones and antagonists, and miscellaneous agents such as platinum coordination complexes, anthracendiones, substituted ureas, etc. Those of skill in the art will know of other chemotherapeutic agents.

Preferred chemotherapeutic agents are those, which in the free form, demonstrate unacceptable systemic toxicity at desired doses. The general systemic 10 toxicity associated with therapeutic levels of such agents is reduced by their linkage to a megalin ligand or a megalin binding fragment of a megalin ligand. Particularly preferred are cardiotoxic compounds that are useful therapeutics but are dose limited by cardiotoxicity. A classic example is adriamycin (also known as doxorubicin) and its analogs, such as daunorubicin. Linking a megalin ligand or a megalin-binding 15 fragment thereof to such drugs decreases accumulation and associated cardiotoxicity at the heart.

VII. METHODS FOR MAKING CONJUGATES

The present invention generally provides methods and compositions 20 comprising megalin ligands or megalin-binding fragments thereof linked to an active agent.

In general, megalin ligand-active agent conjugates can be prepared using techniques known in the art. There are numerous approaches for the conjugation or chemical crosslinking of compounds to proteins and one skilled in the 25 art can determine which method is appropriate for the active agent to be conjugated. The method employed must be capable of joining the active agent to the megalin ligand or megalin-binding fragment thereof without interfering with the ability of the megalin ligand/fragment to bind to megalin, preferably without altering the desired activity of the compound once delivered. Preferred methods of conjugating the ligand 30 to various compounds are set out in the example section, below. Particularly preferred for linking complex molecules to a megalin ligand, such as RAP, is the SATA/sulfo-SMCC cross-linking reaction (Pierce, Rockford, IL). For linking metals

to megalin ligand, preferred reactions include, but are not limited to, binding to tyrosine residues through Chloramine T methods, or use of Iodo beads (Pierce) for iodination reactions.

Methods for conjugating the megalin ligand with the representative labels set forth above may be readily accomplished by one of ordinary skill in the art (see, Trichothecene Antibody Conjugate, U.S. Patent No. 4,744,981; Antibody Conjugate, U.S. Patent No. 5,106,951; Fluorogenic Materials and Labeling Techniques, U.S. Patent No. 4,018,884; Metal Radionuclide Labeled Proteins for Diagnosis and Therapy, U.S. Patent No. 4,897,255; and Metal Radionuclide Chelating Compounds for Improved Chelation Kinetics, U.S. Patent No. 4,988,496; see also Inman, *Methods In Enzymology*, Vol. 34, Affinity Techniques, Enzyme Purification: Part B, Jakoby and Wichek (eds.), Academic Press, New York, p. 30, 1974; see also Wilchek and Bayer, "The Avidin-Biotin Complex in Bioanalytical Applications," *Anal. Biochem.* 171:1-32, 1988; all incorporated herein by reference in their entirety for all purposes).

If the active agent is a protein or a peptide, there are many crosslinkers available in order to conjugate the active agent with the megalin ligand or megalin binding fragment thereof. (See for example, *Chemistry of Protein Conjugation and Crosslinking*, 1991, Shans Wong, CRC Press, Ann Arbor). The crosslinker is generally chosen based on the reactive functional groups available or inserted on the therapeutic compound. In addition, if there are no reactive groups a photoactivatable crosslinker can be used. In certain instances, it may be desirable to include a spacer between the megalin ligand and the active agent. In one example, megalin ligand and protein therapeutic compounds can be conjugated by the introduction of a sulfhydryl group on the megalin ligand and the introduction of a cross-linker containing a reactive thiol group on to the protein compound through carboxyl groups (see, Wawizynczak and Thorpe, in *Immunoconjugates: Antibody Conjugates in Radioimaging and Therapy of Cancer*, C.W. Vogel (Ed.) Oxford University Press, 1987, pp. 28-55.; and Blair and Ghose, *J. Immunol. Methods* 59:129, 1983).

Ligand-chemotherapeutic agents can comprise one or more compound moieties linked to the megalin ligand or megalin-binding fragment thereof. For example, conjugation reactions may conjugate from 1 to 10 or more molecules of adriamycin to a single megalin ligand molecule. Several atoms of gold or iodine can

- be conjugated to a single megalin ligand or megalin-binding fragment thereof. These formulations can be employed as mixtures, or they may be purified into specific megalin ligand-active compound stoichiometric formulations. Those skilled in the art are able to determine which format and which stoichiometric ratio is preferred.
- 5 Further, mixtures of active compounds may be linked to the megalin ligand or megalin-binding fragment thereof, such as the RAP adriamycin-cisplatinum composition set out in the examples. These megalin ligand-active agent conjugates may consist of a range of stoichiometric ratios of ligand to an active agent (e.g., RAP:active agent ratios of 1:1 to 1:4; 1:5 to 1:10; or 1:10 to 1:20). Optionally, a
- 10 plurality of different active agents (e.g. 2, 3, or 4 such agents) may be each conjugated to the megalin ligand or megalin-binding fragment thereof in its own stoichiometric ratio such that megalin ligand or megalin-binding fragment thereof to the total ratio of such additional active agents is not fewer than 1 megalin ligand or megalin-binding fragment thereof per 20 active agents. These, too, may be separated into purified
- 15 mixtures or they may be employed in aggregate.

- The linker is preferably an organic moiety constructed to contain an alkyl, aryl and/or amino acid backbone and which will contain an amide, ether, ester, hydrazone, disulphide linkage or any combination thereof. Linkages containing amino acid, ether and amide bound components will be stable under conditions of
- 20 physiological pH, normally 7.4 in serum and 4-5 on uptake into cells (endosomes). Preferred linkages are linkages containing esters or hydrazones that are stable at serum pH but hydrolyse to release the drug when exposed to intracellular pH. Disulphide linkages are preferred because they are sensitive to reductive cleavage; amino acid linkers can be designed to be sensitive to cleavage by specific enzymes in
- 25 the desired target organ. Exemplary linkers are set out in Blattler et al. *Biochem.* 24:1517-1524, 1985; King et al. *Biochem.* 25:5774-5779, 1986; Srinivasachar and Nevill, *Biochem.* 28:2501-2509, 1989.

- Drug-Linker intermediates are similar to what has been described above but with either an active ester to react with free amine groups on the megalin
- 30 ligand or megalin-binding fragment thereof or a maleimide to react with the free thiols that have been created on the megalin ligand or megalin-binding fragment thereof through other groups where persons skilled in the art can attach them to megalin ligand or megalin-binding fragment thereof.

Methods of crosslinking proteins and peptides are well known to those of skill in the art. Several hundred crosslinkers are available for conjugating a compound of interest with a polypeptide such as a megalin ligand or megalin-binding fragment thereof or with a substance which binds such as a ligand (see, e.g.,

5 Chemistry of Protein Conjugation and Crosslinking, Shans Wong, CRC Press, Ann Arbor (1991) and U.S. Patent No. 5,981,194 and PCT Patent Publication Nos. WO 02/13843 and WO 01/59459 which are incorporated herein by reference in their entirety). Many reagents and cross-linkers can be used to prepare conjugates of an active agent and a megalin ligand such as a RAP molecule, for instance, Hermanson

10 et al. Bioconjugate Techniques, Academic Press, (1996). The crosslinker is generally chosen based on the reactive functional groups available or inserted on the therapeutic agent. In addition, if there are no reactive groups, a photoactivatable crosslinker can be used. In certain instances, it may be desirable to include a spacer between megalin ligand and the agent. In one embodiment, megalin ligand and the protein therapeutic

15 agents may be conjugated by the introduction of a sulfhydryl group on megalin ligand and by the introduction of a crosslinker containing a reactive thiol group on to the protein compound through carboxyl groups (Wawizynczak and Thorpe in Immunoconjugates: Antibody Conjugates in Radioimaging and Therapy of Cancer, Vogel (Ed.) Oxford University Press, pp. 28-55 (1987); and Blair and Ghose (1983) J.

20 Immunol. Methods 59:129). In some embodiments, the linker is vulnerable to hydrolysis at the acidic pH of the lysosome so as to free the agent from the and/or linker.

When a linker is used, the linker is preferably an organic moiety constructed to contain an alkyl, aryl and/or amino acid backbone, and containing an

25 amide, ether, ester, hydrazone, disulphide linkage or any combination thereof. Linkages containing amino acid, ether and amide bound components are stable under conditions of physiological pH, normally 7.4 in serum. Preferred linkages are those containing esters or hydrazones that are stable at serum pH, but that hydrolyze to release the drug when exposed to lysosomal pH. Disulphide linkages are preferred

30 because they are sensitive to reductive cleavage. In addition, amino acid linkers may be designed to be sensitive to cleavage by specific enzymes in the desired target organ or more preferably, the lysosome itself. Exemplary linkers are described in Blattler et

al. (1985) *Biochem.* 24:1517-1524; King et al. (1986) *Biochem.* 25:5774-5779; Srinivasachar and Nevill (1989) *Biochem.* 28:2501-2509.

In some embodiments, the linker is a polyethylene glycol or polypropylene glycol. In other embodiments, the linker is from 4 to 20 atoms long.

5 In other embodiments, the linker is from 1 to 30 atoms long with carbon chain atoms that may be substituted by heteroatoms independently selected from the group consisting of O, N, or S. In some embodiments, from 1 to 4 or up to one-third of the C atoms are substituted with a heteroatom independently selected from O, N, S. In other embodiments, the linker contains a moiety subject to hydrolysis upon delivery

10 to the lysosomal environment (e.g., susceptible to hydrolysis at the lysosomal pH or upon contact to a lysosomal enzyme). In some embodiments, the linker group is preferably hydrophilic to enhance the solubility of the conjugate in body fluids. In some embodiments, the linker contains or is attached to the megalin ligand molecule or the protein agent by a functional group subject to attack by other lysosomal

15 enzymes (e.g., enzymes not deficient in the target lysosome or a lysosomal enzyme not conjugated to the megalin ligand carrier). In some embodiments, the megalin ligand and agent are joined by a linker comprising amino acids or peptides, lipids, or sugar residues. In some embodiments, the megalin ligand and agent are joined at groups introduced synthetically or by post-translational modifications.

20 In some embodiments, agent-linker intermediates are similar to what has been described previously, but comprise, for example, either an active ester that can react with free amine groups on megalin ligand or a maleimide that can react with the free thiols created on megalin ligand via a SATA reaction or through other groups to which the active agent may be attached.

25 **A. Methods for Conjugating a Megalin Ligand Polypeptide to a Protein or Enzyme.**

One of ordinary skill in the art would know how to conjugate an active agent to a protein or peptide. Methods of conjugating active agents and labels to proteins are well known in the art. See, for instance, U.S. Patent No. 5,981,194.

30 Many reagents and cross linkers can be used to prepare bioconjugates of an active agent and a biopolymer. See, for instance, Hermanson et al. *Bioconjugate Techniques*, Academic Press, (1996).

In some embodiments of the present invention, the megalin ligand and the active agent are both polypeptides and the megalin ligand-active agent conjugate is a fusion protein. Fusion proteins may be prepared using standard techniques known in the art. Typically, a DNA molecule encoding the megalin ligand or a portion thereof is linked to a DNA molecule encoding the protein compound. The chimeric DNA construct, along with suitable regulatory elements can be cloned into an expression vector and expressed in a suitable host. The resultant fusion proteins contain megalin ligand or a portion thereof used to the selected protein compound. Megalin ligand-LSD enzyme proteins are particularly contemplated, and exemplary such conjugates include the RAP-human alpha glucosidase and RAP-iduronidase conjugates/fusion proteins described in Example VII and Figures 3 and 4. These fusion proteins were prepared using standard techniques known in the art.

The chimeric protein of the present invention can be produced using host cells expressing a single nucleic acid encoding the entire chimeric protein or more than one nucleic acid sequence, each encoding a domain of the chimeric protein and, optionally, an amino acid or amino acids which will serve to link the domains. The chimeric proteins can also be produced by chemical synthesis.

Host Cells

Host cells used to produce chimeric proteins are bacterial, yeast, insect, non-mammalian vertebrate, or mammalian cells; the mammalian cells include, but are not limited to, hamster, monkey, chimpanzee, dog, cat, bovine, porcine, mouse, rat, rabbit, sheep and human cells. The host cells can be immortalized cells (a cell line) or non-immortalized (primary or secondary) cells and can be any of a wide variety of cell types, such as, but not limited to, fibroblasts, keratinocytes, epithelial cells (e.g., mammary epithelial cells, intestinal epithelial cells), ovary cells (e.g., Chinese hamster ovary or CHO cells), endothelial cells, glial cells, neural cells, formed elements of the blood (e.g., lymphocytes, bone marrow cells), muscle cells, hepatocytes and precursors of these somatic cell types. Host cells can include mutants of CHO cells that do not express LRP such as CHO13-5-1 (FitzGerald et al., J. Biol. Chem., 129(6):1533-41, 1995).

Cells that contain and express DNA or RNA encoding the chimeric protein are referred to herein as genetically modified cells. Mammalian cells that

contain and express DNA or RNA encoding the chimeric protein are referred to as genetically modified mammalian cells. Introduction of the DNA or RNA into cells is by a known transfection method, such as electroporation, microinjection, microprojectile bombardment, calcium phosphate precipitation, modified calcium phosphate precipitation, cationic lipid treatment, photoporation, fusion methodologies, receptor mediated transfer, or polybrene precipitation. Alternatively, the DNA or RNA can be introduced by infection with a viral vector. Methods of producing cells, including mammalian cells, which express DNA or RNA encoding a chimeric protein are described in co-pending patent applications U.S. Ser. No. 08/334,797, entitled "In Vivo Protein Production and Delivery System for Gene Therapy", by Richard F Selden, Douglas A. Treco and Michael W. Heartlein (filed Nov. 4, 1994); U.S. Ser. No. 08/334,455, entitled "In Vivo Production and Delivery of Erythropoietin or Insulinotropin for Gene Therapy", by Richard F Selden, Douglas A. Treco and Michael W. Heartlein (filed Nov. 4, 1994) and U.S. Ser. No. 08/231,439, entitled "Targeted Introduction of DNA Into Primary or Secondary Cells and Their Use for Gene Therapy", by Douglas A. Treco, Michael W. Heartlein and Richard F Selden (filed Apr. 20, 1994). The teachings of each of these applications are expressly incorporated herein by reference in their entirety.

Nucleic Acid Constructs

A nucleic acid construct used to express the chimeric protein can be one which is expressed extrachromosomally (episomally) in the transfected mammalian cell or one which integrates, either randomly or at a pre-selected targeted site through homologous recombination, into the recipient cell's genome. A construct which is expressed extrachromosomally comprises, in addition to chimeric protein-encoding sequences, sequences sufficient for expression of the protein in the cells and, optionally, for replication of the construct. It typically includes a promoter, chimeric protein-encoding DNA and a polyadenylation site. The DNA encoding the chimeric protein is positioned in the construct in such a manner that its expression is under the control of the promoter. Optionally, the construct may contain additional components such as one or more of the following: a splice site, an enhancer sequence, a selectable marker gene under the control of an appropriate promoter, and an amplifiable marker gene under the control of an appropriate promoter.

In those embodiments in which the DNA construct integrates into the cell's genome, it need include only the chimeric protein-encoding nucleic acid sequences. Optionally, it can include a promoter and an enhancer sequence, a polyadenylation site or sites, a splice site or sites, nucleic acid sequences which
5 encode a selectable marker or markers, nucleic acid sequences which encode an amplifiable marker and/or DNA homologous to genomic DNA in the recipient cell to target integration of the DNA to a selected site in the genome (targeting DNA or DNA sequences).

10 Cell Culture Methods

Mammalian cells containing the chimeric protein-encoding DNA or RNA are cultured under conditions appropriate for growth of the cells and expression of the DNA or RNA. Those cells which express the chimeric protein can be identified, using known methods and methods described herein, and the chimeric protein isolated
15 and purified, using known methods and methods also described herein; either with or without amplification of chimeric protein production. Identification can be carried out, for example, through screening genetically modified mammalian cells displaying a phenotype indicative of the presence of DNA or RNA encoding the chimeric protein, such as PCR screening, screening by Southern blot analysis, or screening for
20 the expression of the chimeric protein. Selection of cells having incorporated chimeric protein-encoding DNA may be accomplished by including a selectable marker in the DNA construct and culturing transfected or infected cells containing a selectable marker gene under conditions appropriate for survival of only those cells that express the selectable marker gene. Further amplification of the introduced DNA
25 construct can be affected by culturing genetically modified mammalian cells under conditions appropriate for amplification (e.g., culturing genetically modified mammalian cells containing an amplifiable marker gene in the presence of a concentration of a drug at which only cells containing multiple copies of the amplifiable marker gene can survive).

30 Genetically modified mammalian cells expressing the chimeric protein can be identified, as described herein, by detection of the expression product. For example, mammalian cells expressing chimeric protein in which the carrier is a

megalín ligand can be identified by a sandwich enzyme immunoassay. The antibodies can be directed toward the megalín-binding portion or the active agent portion of the conjugate.

5 VIII. LABELS

In some embodiments, the megalín ligand based active agent conjugate is labeled to facilitate its detection. A "label" or a "detectable moiety" is a composition detectable by spectroscopic, photochemical, biochemical,
10 immunochemical, chemical, or other physical means. For example, labels suitable for use in the present invention include, for example, radioactive labels (e.g., ^{32}P), fluorophores (e.g., fluorescein), electron dense reagents, enzymes (e.g., as commonly used in an ELISA), biotin, digoxigenin, or haptens and proteins which can be made detectable, e.g., by incorporating a radiolabel into the hapten or peptide, or used to detect antibodies specifically reactive with the hapten or peptide.

15 As noted above, depending on the screening assay employed, the active agent, the linker or the megalín ligand polypeptide portion of a conjugate may be labeled. The particular label or detectable group used is not a critical aspect of the invention, as long as it does not significantly interfere with the biological activity of the conjugate. The detectable group can be any material having a detectable physical
20 or chemical property. Thus, a label is any composition detectable by spectroscopic, photochemical, biochemical, immunochemical, electrical, optical or chemical means.

Examples of labels suitable for use in the present invention include, but are not limited to, fluorescent dyes (e.g., fluorescein isothiocyanate, Texas red, rhodamine, and the like), radiolabels (e.g., ^3H , ^{125}I , ^{35}S , ^{14}C , or ^{32}P), enzymes (e.g.,
25 horse radish peroxidase, alkaline phosphatase and others commonly used in an ELISA), and colorimetric labels such as colloidal gold or colored glass or plastic beads (e.g., polystyrene, polypropylene, latex, etc.).

The label may be coupled directly or indirectly to the desired component of the assay according to methods well known in the art. Preferably, the
30 label in one embodiment is covalently bound to the biopolymer using an isocyanate reagent for conjugating an active agent according to the invention. In one aspect of the invention, the bifunctional isocyanate reagents of the invention can be used to

conjugate a label to a biopolymer to form a label biopolymer conjugate without an active agent attached thereto. The label biopolymer conjugate may be used as an intermediate for the synthesis of a labeled conjugate according to the invention or may be used to detect the biopolymer conjugate. As indicated above, a wide variety of labels can be used, with the choice of label depending on sensitivity required, ease of conjugation with the desired component of the assay, stability requirements, available instrumentation, and disposal provisions. Non-radioactive labels are often attached by indirect means. Generally, a ligand molecule (e.g., biotin) is covalently bound to the molecule. The ligand then binds to another molecules (e.g., streptavidin) molecule, which is either inherently detectable or covalently bound to a signal system, such as a detectable enzyme, a fluorescent compound, or a chemiluminescent compound.

The conjugates can also be conjugated directly to signal generating compounds, e.g., by conjugation with an enzyme or fluorophore. Enzymes suitable for use as labels include, but are not limited to, hydrolases, particularly phosphatases, esterases and glycosidases, or oxidotases, particularly peroxidases. Fluorescent compounds, i.e., fluorophores, suitable for use as labels include, but are not limited to, fluorescein and its derivatives, rhodamine and its derivatives, dansyl, umbelliferone, etc. Further examples of suitable fluorophores include, but are not limited to, eosin, TRITC-amine, quinine, fluorescein W, acridine yellow, lissamine rhodamine, B sulfonyl chloride erythroscein, ruthenium (tris, bipyridinium), Texas Red, nicotinamide adenine dinucleotide, flavin adenine dinucleotide, etc. Chemiluminescent compounds suitable for use as labels include, but are not limited to, luciferin and 2,3-dihydrophthalazinediones, e.g., luminol. For a review of various labeling or signal producing systems that can be used in the methods of the present invention, see U.S. Patent No. 4,391,904.

Means of detecting labels are well known to those of skill in the art. Thus, for example, where the label is a radioactive label, means for detection include a scintillation counter or photographic film as in autoradiography. Where the label is a fluorescent label, it may be detected by exciting the fluorochrome with the appropriate wavelength of light and detecting the resulting fluorescence. The fluorescence may be detected visually, by the use of electronic detectors such as charge coupled devices (CCDs) or photomultipliers and the like. Similarly,

enzymatic labels may be detected by providing the appropriate substrates for the enzyme and detecting the resulting reaction product. Colorimetric or chemiluminescent labels may be detected simply by observing the color associated with the label. Other labeling and detection systems suitable for use in the methods of the present invention will be readily apparent to those of skill in the art. Such labeled modulators and ligands may be used in the diagnosis of a disease or health condition.

VIII. SCREENING ASSAYS FOR MEGALIN LIGAND-ACTIVE AGENT CONJUGATES AND MODULATORS OF THEIR DELIVERY

The present invention provides a screening assay for megalin ligand polypeptide-active agent conjugates, wherein the conjugates are tested for their ability to influence a measurable activity of the megalin receptor which can be situated in a whole cell, a cell extract, semi-purified, purified or any other format that allows for measurement of its activity. The activity can be any activity in the expression, function or degradation of megalin including, for example, the amount or timing of such activities. Such activities include, for example, transcription, transcript processing, translation or transcript stability of the megalin gene sequence or mRNA transcript. Such activities include, for example, the synthesis of new LRP, the sub-cellular localization of megalin and activation of megalin biological activity. Such activities include, for example, the ability of megalin to bind substances, adopt conformations, catalyze reactions, bind known ligands and the like. Such activities include, for example, the amount or stability of megalin, the processing and removal or degradation of megalin and the like. In preferred embodiments, the megalin ligand used is one which has been modified or naturally has a higher binding affinity for megalin than for any other LRP receptor, and particularly a higher binding affinity for megalin than for LRP1. Screening assays similar to those discussed above for megalin may be set up for any other LRP receptors to yield a comparison of the relative binding affinities of the megalin ligand for megalin as compared to other LRP receptors.

The invention contemplates a variety of different screening formats. Some designs are considered low throughput and test only one or a few compounds in series or in parallel. High throughput screening assays are suitable for screening tens

of thousands or hundreds of thousands of compounds in a matter of weeks or months. "In silico" screening formats employ computer-aided rational design techniques to identify potential modulators of megalin biological activity.

A. Modulating Uptake of Megalin Ligand-Conjugated Active Agents by Modulating Megalin Receptor Activity

5

Those skilled in the art will appreciate that increasing megalin ligand-active agent conjugate uptake and delivery to targets including, but not limited to, the brain or lysosomes is useful and desirable in situations such as, but not limited to, where the conjugate is being used to treat a neurological condition and/or a LSD and increased amounts of delivery would provide therapeutic benefit. Those skilled in the art will appreciate that decreasing conjugate uptake and delivery across the blood-brain barrier is useful and desirable for a variety of reasons including, but not limited to, where the conjugate is being used for its potential cardio-protective effect or used in other (non-CNS) organs and side-effects of brain uptake are to be avoided.

15

Suitable megalin ligands, megalin-binding fragment thereof, active agent conjugates of megalin ligands or megalin-binding fragment thereof, and modulators of megalin and/or other LRP activity and modulators of megalin ligand conjugate delivery can also be readily identified using a modification of the Transwell apparatus set out in EXAMPLE 1 below. In the modified form, a compound (e.g., megalin ligand, a conjugate of a megalin ligand with an active agent or a modulator) is added to the luminal surface of the cells in the Transwell apparatus. The compound is then scored according to how well it is able to traverse across the BBCECs to the abluminal side or as to how well (if a modulator) it increases or decreases the transport of a megalin ligand or a megalin binding fragment of a megalin ligand or another LRP ligand across the BBCECs to the abluminal side. A library of compounds can be readily screened or tested to identify pharmacologically superior modulators.

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An exemplary ligand used herein is RAP. Other known ligands of the megalin receptor may be screened for use as modulators of the delivery of the conjugate, or as models for designing such modulators. These ligands include, but are not limited to, ApoE, Chylomicron remnants, β -VLDL, activated α 2-macroglobulin, tPA, Tissue factor inhibitor, Pro-uPA, PAI-1, Saposin, Gentamycin, Thyroglobulin,

30

Polymixin B, Seminal Vesicle Secretory Protein A, Thrombospondin -1, Lactoferrin, and β -APP. These ligands may be modified to increase their binding affinity to megalin. Those ligands with a greater binding affinity to megalin as compared to LRP1 are particularly preferred.

5

IX. METHODS OF USING, PHARMACEUTICAL COMPOSITIONS, AND THEIR ADMINISTRATION

The conjugates and modulators may be administered by a variety of routes. For oral preparations, the conjugates can be used alone or in combination with appropriate additives to make tablets, powders, granules or capsules, for example, with conventional additives, such as lactose, mannitol, corn starch or potato starch; with binders, such as crystalline cellulose, cellulose derivatives, acacia, corn starch or gelatins; with disintegrators, such as corn starch, potato starch or sodium carboxymethylcellulose; with lubricants, such as talc or magnesium stearate; and if desired, with diluents, buffering agents, moistening agents, preservatives and flavoring agents.

The conjugates and modulators can be formulated into preparations for injection by dissolving, suspending or emulsifying them in an aqueous or nonaqueous solvent, such as vegetable or other similar oils, synthetic aliphatic acid glycerides, esters of higher aliphatic acids or propylene glycol; and if desired, with conventional additives such as solubilizers, isotonic agents, suspending agents, emulsifying agents, stabilizers and preservatives.

The conjugates, modulators, and LRP ligands can be utilized in aerosol formulation to be administered via inhalation. The compounds of the present invention can be formulated into pressurized acceptable propellants such as dichlorodifluoromethane, propane, nitrogen and the like.

Furthermore, the conjugates and modulators can be made into suppositories by mixing with a variety of bases such as emulsifying bases or water-soluble bases. The compounds of the present invention can be administered rectally via a suppository. The suppository can include vehicles such as cocoa butter, carbowaxes and polyethylene glycols, which melt at body temperature, yet are solidified at room temperature.

Unit dosage forms of the conjugate, modulator, and LRP ligand for oral or rectal administration such as syrups, elixirs, and suspensions may be provided wherein each dosage unit, for example, teaspoonful, tablespoonful, tablet or suppository, contains a predetermined amount of the composition containing active agent. Similarly, unit dosage forms for injection or intravenous administration may comprise the conjugate in a composition as a solution in sterile water, normal saline or another pharmaceutically acceptable carrier.

In practical use, the conjugate, modulator, and LRP ligand according to the invention can be combined as the active ingredient in intimate admixture with a pharmaceutical carrier according to conventional pharmaceutical compounding techniques. The carrier may take a wide variety of forms depending on the form of preparation desired for administration, e.g., oral or parenteral (including intravenous). In preparing the compositions for oral dosage form, any of the usual pharmaceutical media may be employed, such as, for example, water, glycols, oils, alcohols, flavoring agents, preservatives, coloring agents and the like in the case of oral liquid preparations, such as, for example, suspensions, elixirs and solutions; or carriers such as starches, sugars, microcrystalline cellulose, diluents, granulating agents, lubricants, binders, disintegrating agents and the like in the case of oral solid preparations such as, for example, powders, hard and soft capsules and tablets, with the solid oral preparations being preferred over the liquid preparations.

With respect to transdermal routes of administration, methods for transdermal administration of drugs are disclosed in Remington's Pharmaceutical Sciences, 17th Edition, (Gennaro et al. Eds. Mack Publishing Co., 1985). Dermal or skin patches are a preferred means for transdermal delivery of the conjugates, modulators, and LRP ligands of the invention. Patches preferably provide an absorption enhancer such as DMSO to increase the absorption of the compounds. Other methods for transdermal drug delivery are disclosed in U.S. Patents No. 5,962,012, 6,261,595, and 6,261,595. Each of which is incorporated by reference in its entirety.

In specific embodiments, it is contemplated that the therapeutic administering of the conjugates described herein will be administered intrathecally into the CSF. The intrathecal administration of the present invention may comprise introducing the pharmaceutical composition into a cerebral ventricle. Alternatively,

the intrathecal administration may comprise introducing the pharmaceutical composition into the lumbar area. In yet another alternative, the intrathecal administration comprises introducing the pharmaceutical composition into the cisterna magna. Any such administration is preferably via a bolus injection. Depending on the severity of the symptoms and the responsiveness of the subject to the therapy, such a bolus injection may be administered once per week, once per month, once every 6 months or annually. In other embodiments, the intrathecal administration is achieved by use of an infusion pump. The pharmaceutical could of course be intrathecally administered continually over a period of at least several days or alternatively, the intrathecal administration is continually over a period of at least four weeks. Of course, where the administration is via continuous infusion, the rate of dose administration of the enzyme replacement therapy may be greatly reduced as compared to the bolus injection administration. In preferred embodiments, the active agent of the conjugate is iduronidase and it is delivered in an amount that comprises about 1 mg iduronidase/20 kg of body weight of the mammal being treated for MPS. In particular embodiments, the above dose is delivered to 15 cc CSF. At such a concentration it is contemplated that the enzyme concentration will be 18,000 units per ml of CSF. It should be understood that the aforementioned dosage is merely an exemplary dosage and those of skill in the art will understand that this dosage may be varied.

The methods and compositions of the invention may be combined with methods and compositions of inducing antigen specific tolerance prior to the enzyme replacement therapy. Such methods include inducing antigen specific tolerance comprises administration of an immunosuppressive agent, such as e.g., cyclosporine A and may further comprise administration of an antiproliferative agent, including but not limited to a nucleotide analog or an anti-metabolite. The antiproliferative agent may be azathioprine. Further methods are described in e.g., U.S. Patent Application No. 10/141,668, published as U.S. Publication No. 20030211113; and U.S. Patent Application No. 10/429,314 published as U.S. Publication No. 20040009906, each incorporated herein by reference.

Pharmaceutically acceptable excipients, such as vehicles, adjuvants, carriers or diluents, are commercially available. Moreover, pharmaceutically

acceptable auxiliary substances, such as pH adjusting and buffering agents, tonicity adjusting agents, stabilizers, wetting agents and the like, are commercially available.

Those of skill will readily appreciate that dose levels can vary as a function of the specific compound, the severity of the symptoms and the susceptibility of the subject to side effects. Preferred dosages for a given compound are readily determinable by those of skill in the art by a variety of means, including, but not limited to dose response and pharmacokinetic assessments conducted in patients, test animals, and in vitro.

In each of these aspects, the compositions include, but are not limited to, compositions suitable for oral, rectal, topical, parenteral (including subcutaneous, intramuscular, and intravenous), pulmonary (nasal or buccal inhalation), or nasal administration, although the most suitable route in any given case will depend in part on the nature and severity of the conditions being treated and on the nature of the active ingredient. Exemplary routes of administration are the oral and intravenous routes. The compositions may be conveniently presented in unit dosage form and prepared by any of the methods well-known in the art of pharmacy.

In practical use, the modulators or according to the invention can be combined as the active ingredient in intimate admixture with a pharmaceutical carrier according to conventional pharmaceutical compounding techniques. The carrier may take a wide variety of forms depending on the form of preparation desired for administration, e.g., oral or parenteral (including intravenous). In preparing the compositions for oral dosage form, any of the usual pharmaceutical media may be employed, such as, for example, water, glycols, oils, alcohols, flavoring agents, preservatives, coloring agents and the like in the case of oral liquid preparations, such as, for example, suspensions, elixirs and solutions; or carriers such as starches, sugars, microcrystalline cellulose, diluents, granulating agents, lubricants, binders, disintegrating agents and the like in the case of oral solid preparations such as, for example, powders, hard and soft capsules and tablets, with the solid oral preparations being preferred over the liquid preparations.

Because of their ease of administration, tablets and capsules represent the most advantageous oral dosage unit form in which case solid pharmaceutical carriers are obviously employed. If desired, tablets may be coated by standard

aqueous or nonaqueous techniques. The percentage of an active compound in these compositions may, of course, be varied and may conveniently be between about 2 percent to about 60 percent of the weight of the unit.

5 The conjugates, modulators, and ligands of the invention are useful for therapeutic, prophylactic and diagnostic intervention in animals, and in particular in humans. As described herein, the conjugates show preferential accumulation and/or release of the active agent in any target organ, compartment, or site depending upon the biopolymer used.

Compositions of the present invention may be administered
10 encapsulated in or attached to viral envelopes or vesicles, or incorporated into cells. Vesicles are micellular particles which are usually spherical and which are frequently lipidic. Liposomes are vesicles formed from a bilayer membrane. Suitable vesicles include, but are not limited to, unilamellar vesicles and multilamellar lipid vesicles or liposomes. Such vesicles and liposomes may be made from a wide range of lipid or
15 phospholipid compounds, such as phosphatidylcholine, phosphatidic acid, phosphatidylserine, phosphatidylethanolamine, sphingomyelin, glycolipids, gangliosides, etc. using standard techniques, such as those described in, e.g., U.S. Patent No. 4,394,448. Such vesicles or liposomes may be used to administer compounds intracellularly and to deliver compounds to the target organs. Controlled
20 release of a p97-composition of interest may also be achieved using encapsulation (see, e.g., U.S. Patent No. 5,186,941).

Any route of administration that delivers the megalin ligand-based active agent conjugate or modulator composition into the blood stream, or preferably at least outside of the blood-brain barrier, may be used. Preferably, the composition is
25 administered peripherally, most preferably intravenously or by cardiac catheter. Intrajugular and intracarotid injections are also useful. Compositions may be administered locally or regionally, such as intraperitoneally or subcutaneously or intramuscularly. In one aspect, compositions are administered with a suitable pharmaceutical diluent or carrier.

30 Dosages to be administered will depend on individual needs, on the desired effect, the active agent used, the biopolymer and on the chosen route of administration. Preferred dosages of a conjugate range from about 0.2 pmol/kg to

about 25 nmol/kg, and particularly preferred dosages range from 2-250 pmol/kg; alternatively, preferred doses of the conjugate may be in the range of 0.02 to 2000 mg/kg. These dosages will be influenced by the number of active agent or drug moieties associated with the biopolymer. Alternatively, dosages may be calculated
5 based on the active agent administered.

In preferred embodiments the conjugate comprises human RAP. For instance, doses of RAP-adriamycin comprising from 0.005 to 100 mg/kg of adriamycin are also useful in vivo. Particularly preferred is a dosage of RAP-adriamycin comprising from 0.05 mg/kg to 20 mg/kg of adriamycin. Those skilled in
10 the art can determine suitable doses for compounds linked to a megalin ligand based in part on the recommended dosage used for the free form of the compound. Conjugation of the active agent to a megalin ligand such as RAP generally reduces the amount of drug needed to obtain the same effect.

The conjugates and modulators of the invention are useful for
15 therapeutic, prophylactic and diagnostic intervention in animals, and in particular in humans. Megalin ligand compounds may show preferential accumulation in particular tissues. Preferred medical indications for diagnostic uses include, for example, any condition associated with a target organ of interest (e.g., lung, liver, kidney, spleen). In particularly preferred embodiments, the target organ of interest in
20 the brain.

The subject methods find use in the treatment of a variety of different disease conditions. In certain embodiments, of particular interest is the use of the subject methods in disease conditions where an active agent or drug having desired activity has been previously identified, but in which the active agent or drug is not
25 adequately delivered to the target site, area or compartment to produce a fully satisfactory therapeutic result. With such active agents or drugs, the subject methods of conjugating the active agent to a megalin ligand or a megalin binding fragment thereof can be used to enhance the therapeutic efficacy and therapeutic index of active agent or drug.

30 The specific disease conditions treatable by with the subject conjugates are as varied as the types of drug moieties that can be present in the conjugate. Thus, disease conditions include cellular proliferative diseases, such as neoplastic diseases,

autoimmune diseases, cardiovascular diseases, hormonal abnormality diseases, degenerative diseases, diseases of aging, diseases of the central nervous system (e.g., Alzheimer's disease, epilepsy, hyperlipidemias), psychiatric diseases and conditions (e.g., schizophrenia, mood disorders such as depression and anxiety), infectious
5 diseases, enzyme deficiency diseases, lysosomal storage diseases such as those described above, and the like.

Treatment is meant to encompass any beneficial outcome to a subject associated with administration of a conjugate including a reduced likelihood of acquiring a disease, prevention of a disease, slowing, stopping or reversing, the
10 progression of a disease or an amelioration of the symptoms associated with the disease condition afflicting the host, where amelioration or benefit is used in a broad sense to refer to at least a reduction in the magnitude of a parameter, e.g., symptom, associated with the pathological condition being treated, such as inflammation and pain associated therewith. As such, treatment also includes situations where the
15 pathological condition, or at least symptoms associated therewith, are completely inhibited, e.g., prevented from happening, or stopped, e.g., terminated, such that the host no longer suffers from the pathological condition, or at least the symptoms that characterize the pathological condition.

In specific embodiments, the disorder being treated is a lysosomal
20 storage disease and the conjugate is administered as a pharmaceutical composition in an amount effective to decrease the amount of storage granules present in the brain tissue of said mammal. Typically, the symptoms of such a disorder are monitored through routine assessment of history, physical examination, echocardiography, electrocardiography, magnetic resonance imaging, polysomnography, skeletal survey,
25 range of motion measurements, corneal photographs, and skin biopsy. Administration of a megalin-binding moiety conjugated to a therapeutic agent in such a disorder results in normalization of developmental delay and regression in said subject, reduction in high pressure hydrocephalus, reduction in spinal cord compression in said subject, and reduction in number and/or size of perivascular cysts around the
30 brain vessels of said subject. Methods of monitoring and assessing such sequelae are known to those of skill in the art. Those of skill in the art are referred to U.S. Patent No. 6,585,971; U.S. Patent No. 6,569,661 and U.S. Patent No. 6,426,208 and U.S. Patent Publication No. 20040009906 for additional descriptions of such sequelae.

In some aspects, it may be useful to increase the tolerance of the animal to the therapy being delivered. Such methods are described in U.S. Patent Application No. 10/429,314 filed May 5, 2003 and published as 20040009906 (incorporated herein by reference in its entirety).

5 In preferred embodiments, the animal is suffering from mucopolysaccharidosis I and has about 50% or less of a normal α -L-iduronidase activity. In such embodiments, it would be desirable to administered an effective dose of between about 0.001mg/kg body weight and 0.5 mg/kg body weight of human α -L-iduronidase as part of the conjugate e.g., weekly to a subject suffering from a
10 deficiency thereof. In other embodiments, the subject is given a dose of between about 0.01 mg/15 cc of CSF to about 5.0 mg/15 cc of CSF in the mammal of said human α -L-iduronidase weekly. The therapies contemplated herein promote the breakdown of glycosaminoglycan (GAG) in a brain cell of a subject having lysosomal storage disease. The brain cell may be a neuron, a neuroglial cell, an ependymal cell.
15 Typically, the brain cells in which granule accumulation occurs and should be ameliorated by administering a conjugate of the invention include neurons, glial cells, microglial cells, astrocytes, oligodendroglial cells, perivascular cells, perithelial cells, meningeal cells, ependymal cells, arachnoid granulation cells, arachnoid membranes, dura mater, pia mater and choroid plexus cells. The therapy in preferred
20 embodiments reduces storage granules in meningeal cells as compared to the number of lysosomal storage granules present in a similar cell in the absence of administration of said conjugate. This produces the therapeutic effects of relieving the symptoms of high pressure hydrocephalus in some subjects. and said administering reduces the amount of CSF fluid in the meningeal tissue of said subject.

25 A variety of hosts or subjects are treatable according to the subject methods. Generally such hosts are "mammals" or "mammalian," where these terms are used broadly to describe organisms which are within the class mammalia, including the orders carnivore (e.g., dogs and cats), rodentia (e.g., mice, guinea pigs, and rats), and primates (e.g., humans, chimpanzees, and monkeys). In many
30 embodiments, the hosts will be humans.

XI. PRODUCTION OF MEGALIN LIGAND POLYPEPTIDES

In the present invention, numerous megalin ligands may be used to facilitate transcytosis of a given active agent. One exemplary such ligand is RAP. RAP and RAP polypeptides for use according to the invention include those disclosed in U.S. Patent No. 5,474,766 that is enclosed herein by reference in its entirety for the purposes of disclosing such peptides and how they may be obtained for use in the compounds and compositions of the present invention. RAP, and RAP polypeptides, and other megalin ligands may be produced using any of protein preparation and purification methods known to those of skill in the art.

The ligand can be purified from a naturally occurring source of the protein, can be isolated from a recombinant host expressing the ligand, or can be synthesized using well known techniques in protein synthesis. A skilled artisan can readily adapt a variety of such techniques in order to obtain a megalin ligand that contain the megalin binding site. Such a megalin ligand may for example possess the megalin docking/binding site found on RAP. See, for instance, Melman et al., *J. Biol. Chem.* 276 (31): 29338-29346 (2001); Savonen et al., *J Biol Chem.* 274(36): 25877-25882 (1999); Nielsen et al. *Proc. Natl. Acad. Sci. USA* 94:7521-7525 (1997); Medved et al., *J. Biol. Chem.* 274(2): 717-727 (1999); Rall et al., *J. Biol. Chem.* 273(37): 24152-24157 (1998); Orlando et al., *Proc. Natl. Acad. Sci. USA* 3161-3163 (1994).

The isolation of native RAP proteins has been described in Ashcom et al., *J. Cell. Biol.* 110:1041-1048 (1990) and Jensen et al., *FEBS Lett.* 255:275-280 (1989). Megalin ligand fragments containing the megalin binding site may be generated from isolated native protein which is converted by enzymatic and/or chemical cleavage to generate fragments of the whole protein. Exemplary such methods are taught in U.S. Patent No. 6,447,775 which is herein incorporated by reference with particular reference to such methods for obtaining RAP polypeptides.

In addition, the megalin ligand or a megalin binding fragment of such a ligand can be expressed in a recombinant bacteria, as described, by Williams et al., *J. Biol. Chem.* 267:9035-9040 (1992) and Wurshawsky et al., *J. Biol. Chem.* 269:3325-3330 (1994).

As indicated herein throughout, RAP is a preferred megalin ligand. Procedures for purifying the 39 kDa RAP protein from a recombinant *E. coli* strain

has been previously described by Herz et al., J. Biol. Chem. 266, 21232-21238 (1991). A modified version of that procedure can be used as described in U.S. Patent No. 5,474,766 and below.

5 Cultures of *E. coli* strain DH5alpha carrying the expression plasmid pGEX-39 kDa can be grown to mid-log phase in LB medium with 100 µg/ml ampicillin at 37°C. Cultures can then be cooled to 30°C and supplemented with 0.01% isopropylthio-beta-D-galactoside to induce expression of the glutathione-S-transferase-39 kDa fusion protein. Following a 4-6 hour induction at 30°C, cultures can be cooled with ice and recovered by centrifugation.

10 All of the following steps are to be carried out at 4°C. Cell pellets are lysed in PBS with 1% Triton X-100, 1 µM pepstatin, 2.5 µg/ml leupeptin, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), and 1 µM ethylenediaminetetraacetate (EDTA). Sonication of this lysate with a Branson Model 450 Sonifier with separation of the resulting membranes and other cellular debris by centrifugation at 15,000 g for
15 15 minutes is then followed by retrieval of the supernatant. The supernatant from this step is incubated overnight with agarose immobilized glutathione beads (Sigma Chemical Co.) in PBS and 0.1% sodium azide. The beads can then be washed, and elution of the fusion protein can be carried out by competition with 5 mM reduced glutathione (Sigma Chemical Co.). Following dialysis, the fusion protein can be
20 cleaved by an overnight incubation with 100 ng of activated human thrombin per 50 µg of fusion protein. The glutathione-S-transferase epitope can subsequently be removed by further incubation with agarose immobilized glutathione beads.

The 28 kDa protein fragment of the 39 kDa protein ("28 kDa protein") of the present invention has the following amino acid sequence set forth in the
25 Sequence Listing as SEQ ID NO:2 (Figure 16).

The 28 kDa protein has a molecular weight of 28,000 daltons on SDS-PAGE, is relatively stable to acid hydrolysis, is soluble in 1% Triton X-100, and has approximately the same inhibitory activity (K_i) on t-PA binding to the hepatic receptor as the 39 kDa protein. The 28kDa protein may be cloned and purified as
30 further exemplified in U.S. Patent No. 5,474,766 which is expressly incorporated herein by reference for such methods of cloning.

While the above method is described for the production and purification of RAP, as indicated above, other megalin ligands and megalin binding fragments also may be produced using similar techniques. A review of such ligands may be found in Christensen and Birn, (Am. J. Physiol. Renal Physiol., 280:F562-F573, 2001, see particularly Table 1 and references cited therein) Techniques for making and purifying such ligands are well known to those of skill in the art.

XIII. EXAMPLES

The following example(s) is included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the example(s) that follows represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention. The following examples provide exemplary protocols for assessing transcytosis in vitro and for characterizing the interaction of megalin ligands such as RAP with megalin and other receptors.

EXAMPLE 1

Transcytosis of p97

Transcytosis experiments were performed as follows. One insert covered with bovine brain capillary endothelial cells (BBCECs) was set into a Transwell apparatus containing a six-well microplate with 2 ml of Ringer/Hepes and pre-incubated for 2 h at 37°C. [¹²⁵I]-p97 (250 nM) was added to the upper side of the filter covered with cells. At various times, the insert was transferred to avoid re-endocytosis of p97 by the abluminal side of the BBCECs. At the end of experiment, [¹²⁵I]-p97 was measured after TCA precipitation. Transcytosis is depicted in Figure 17.

The effect of RAP on transcytosis of ¹²⁵I-p97 was assessed. In Figure 1, RAP, a known polypeptide inhibitor of the LRP family was applied to the cells (25

micrograms/ml). RAP significantly inhibited the transcytosis of p97, thus directly implicating the LRP family in transcytosis.

EXAMPLE 2

5 Construction, expression, purification and characterization of RAP fusions

Expression constructs encoding fusions between the human receptor-associated protein (RAP) and human alpha-glucosidase (GAA), alpha-L-iduronidase (IDU) or glial cell-derived neurotrophic factor (GDNF) were prepared. For this purpose, a sequence that encodes RAP was fused to the 5'-end of sequences that
10 encode the different fusion partners. All sequences were obtained by high-fidelity PCR amplification of human cDNA with the following primers shown in Figure 2a. The GDNF fusion was designed for expression in bacteria. To this end, primer RAPBACF was substituted for RAPF in the RAP amplification for this construct (Figure 2b).

15 The 5'-end of RAP was truncated to remove the signal peptide sequence. Instead, an in-frame BamHI site, which encodes the dipeptide GS, was added for the mammalian expression construct. Sequence encoding the tetrapeptide MGGS with an NcoI site at the 5'-end was added for the bacterial expression construct. The 3'-end of RAP was truncated to remove the tetrapeptide HNEL
20 endoplasmic reticulum retention signal. Instead, the coding sequence for a six amino-acid spacer (AEAETG) was appended. The last two codons of the spacer specify an AgeI restriction site. The 5'-end of GAA was truncated to remove the signal peptide and pro-peptide sequences (Wisselaar, et al., J. Biol. Chem. 268(3):2223-31, 1993). Instead, an AgeI site was added to permit fusion to the RAP-spacer portion of the
25 fusion. The 5'-end of IDU was similarly truncated to remove the signal peptide and introduce the restriction site. The 5'-end of GDNF was truncated to remove both the signal peptide and pro-peptide sequences (Lin et al., Science, 260(5111): 1130-2, 1993).

The open-reading frames encoding the GAA and IDU fusions were
30 ligated into the expression vector pCINmt using flanking BamHI and XhoI sites. The vector contains the human melanotransferrin signal peptide with an in-frame BamHI site at the 3'-end. The sequences of the resulting fusion proteins are shown in Figures

3 and 4. The pCINmt (derived from Invitrogen vector pcDNA3.1) control sequences consist of the human CMV promoter followed by the rabbit IVS2 and the rat preproinsulin RNA leader sequence. A bovine growth hormone terminator sequence is positioned at the 3'-end of the expression cassette. The vector includes a selectable
5 marker composed of an attenuated neomycin phosphotransferase gene driven by the weak HSV-tk promoter (Yenofsky et al., Proc. Nat'l Acad. Sci., USA 87(9):3435-9, 1990). Expression constructs for RAP-GAA and RAP-IDU were transfected into an Lrp-deficient CHO cell line (CHO13-5-1) and recombinants selected with 800µg/mL G418.

10 The RAPGDNF fusion (Figure 5) was cloned into the bacterial expression vector pBADhisA (Invitrogen) using the flanking NcoI and XbaI sites. The resulting expression vector was transfected into BL21 cells and recombinants selected with carbenicillin. Expressed, purified RAP-GDNF fusion may be assayed for the ability to protect dopaminergic neurons or other activities as previously
15 described (Kilic et al., Stroke 34(5):1304-10, 2003).

Expression of RAP fusions

Culture medium was JRH 302 supplemented with 2 mM L-glutamine, gentamycin, amphotericin, 800 µg/mL G418 and 2.5% fetal calf serum. Recombinant
20 clones were grown in T225 flasks prior to seeding into 1 L Corning spinner flasks on Cytopore 1 beads (Amersham) in the presence of serum. Spinner flasks were maintained in a tissue culture incubator set at 37°C and 5% CO₂. Medium was replaced every two days with serum-free medium until serum levels were undetectable. Subsequently, harvests were collected every two days and medium
25 exchanged.

Purification of RAP-GAA for Uptake Assay:

RAP-GAA harvested in the medium from the spinner flasks was applied to a Blue-Sepharose column (Amersham) in low-salt buffer at neutral pH. Fusion was eluted with a linear salt gradient, and fractions containing fusion were
30 loaded to a Heparin-Sepharose column (Amersham) and again eluted with a linear salt gradient. Eluted fractions containing activity were pooled and applied to a Phenyl-Sepharose column (Amersham). RAP-GAA was eluted from the Phenyl-Sepharose

column with a decreasing salt step gradient. Eluted fractions were run on an SDS-PAGE gel and stained to determine relative percent purity. Based on gel analysis, peak activity fractions were about 70% pure. Fractions were pooled, concentrated using a 30kD MWCO membrane (Millipore), and exchanged into phosphate-buffered saline at neutral pH.

The activity of the lysosomal enzyme in the fusion was determined to be unaffected by fusion to RAP. Purified human LRP (1 μ g, recombinant, binding domain 2) was spotted onto PVDF filters in a 96-well dot-blot apparatus. Purified RAP-lysosomal enzyme fusion (RAP-LE) in Tris-buffered saline pH 7.5 with 5 mM CaCl₂ and 3% non-fat dry milk (TBS/Ca/BLOTTO) was overlaid on the immobilized LRP. Conditioned medium containing the RAP-LE, buffer alone and RAP alone were similarly incubated with immobilized LRP. Filters were washed three times to remove unbound protein. Duplicate filters were probed with anti-LE antibody or anti-RAP antibody. Blots were developed with chemiluminescent detection. The activity of the lysosomal enzyme was measured using fluorescent substrates. It was observed as shown in Figure 10 that antibodies to either RAP or to the lysosomal enzyme detect LRP-bound RAP-LE, were found to bind to the fusion on Western blots, indicating that the fused proteins were intact and folded. Comparing signal intensity, it is further observed that the fusion is bound by the immobilized LRP to a similar extent as RAP alone.

Characterization of RAP-GAA fusion:

Purified RAP-GAA was tested to determine identity, purity and carbohydrate content. For the identity test, fusion was resolved on SDS-PAGE, blotted to PVDF and probed with anti-GAA and anti-RAP antibodies. A single band of about 150 kD cross-reacted with both antibodies (Figure 6). Fusion purity was determined by Coomassie Blue staining of the SDS-PAGE gel and was estimated to be >95%. Presence of complex oligosaccharides was measured by digestion with neuraminidase and comparison to undigested samples on an IEF gel. Neuraminidase digestion resulted in a quantitative shift in mobility to a more basic pI, consistent with the presence of complex oligosaccharides (Figure 7). Endo H digestion was used to test for the presence of high-mannose oligosaccharides. Unlike control proteins, no change in molecular weight of the fusion was observed on SDS-PAGE gels after

Endo H digestion. This suggests the absence of high-mannose oligosaccharides on the fusion (Figure 8).

Purification of the RAP-IDU fusion:

Blue sepharose 6 Fast Flow resin is used for the first purification step. The harvest fluid was adjusted to pH 7.0 and loaded onto a Blue-Sepharose column at a 70mL/mL resin basis. The column was equilibrated with 75 mM NaCl, 20 mM Na₂HPO₄ pH 7.0. RAP-IDU eluted off the column at 1.2 M NaCl, 20 mM Na₂HPO₄ pH 7.0. The eluted fraction containing RAP-IDU (determined by iduronidase activity assay) was then exchanged into 75 mM NaCl, 20 mM Na₂PO₄ pH 7.0 and loaded onto a Heparin CL 6B resin. RAP-IDU was eluted from the Heparin column at 0.5 M NaCl pH 7.0. The eluted fusion was then adjusted to 2M NaCl, 20 mM Na₂HPO₄ pH 7.0 and loaded directly onto a Phenyl-Sepharose column. As a final step, RAP-IDU was eluted from this column at between 0.3 to 0.5M NaCl. Fusion purity was estimated by SDS-PAGE at >80% (Figure 9).

EXAMPLE 3**Uptake and distribution of unconjugated RAP to the brain**

The distribution of RAP to brain was measured using a mouse *in situ* perfusion model. Volumes of distribution (V_d) for RAP, the positive control transferrin and the negative control albumin, were determined over a perfusion interval of 5 minutes. In addition, the relative quantities of the test proteins in the vascular and parenchymal fractions of the perfused brain were determined using the capillary depletion technique (Gutierrez et al., J. Neuroimmunol., 47(2):169-76, 1993). The results shown in Figure 11 include an observed, corrected K_{influx} of 1 $\mu\text{L/g/min}$ for transferrin. RAP had an observed, corrected K_{influx} of 2.2 $\mu\text{L/g/min}$. RAP is taken up into brain.

A separate experiment was carried out at a single, 5-minute time-point to determine whether RAP is able to traverse the brain vasculature and enter the parenchyma. Brains were harvested as before, but were subjected to a capillary depletion procedure to determine the levels of RAP and albumin in the vascular and parenchymal spaces. Following harvest, the isolated cortex was weighed and placed in a Dounce homogenizer on ice. The cortex was immediately homogenized in 0.7 ml of capillary buffer (10 mM HEPES, 141 mM NaCl, 4 mM KCl, 2.8 mM CaCl₂, 1 mM NaH₂PO₄, 1 mM MgSO₄, 10 mM glucose, pH 7.4) for 10 strokes, after which 1.7 ml

of 26% dextran was added and the mixture further homogenized with an additional 3 strokes on ice. To separate the different tissue fractions, 1.3 ml of the homogenate was loaded in an ultracentrifuge tube. The homogenate was centrifuged at 9000 rpm (5400 x g) for 15 min at 4°C in a Beckman TLV-100 swinging-bucket rotor. The parenchymal portion (supernatant) and the capillary portion (pellet) were then separately counted in a dual-channel gamma counter. A sample of post-CNS perfusate was also counted for the V_d calculation. Unlabeled RAP was included as a competitor in some cases to determine whether uptake into brain tissue was saturable (5 µg of unlabeled RAP per mouse, about 80-fold excess over labeled RAP). Results were plotted as corrected V_d (Figure 11). Each data point is an average derived from 5-6 mice. Figure 12 shows the distribution of RAP between brain capillary endothelium and brain parenchyma. These results indicate RAP crosses the blood-brain barrier to enter brain parenchyma and that the process of uptake is saturable.

EXAMPLE 4

Measurement of specific uptake of RAP-GAA into enzyme-deficient patient fibroblasts

The uptake of RAGA into cells deficient in GAA was characterized. The cell line used was GM244 (Coriell Cell Repository), a primary cell line isolated from a patient with glycogen storage disorder type II (Pompe's disease). These fibroblasts take up phosphorylated, recombinant GAA via the mannose-6-phosphate receptor, but also have LRP1 receptors, which bind RAP. In order to identify the receptors involved in uptake of different test ligands, samples containing excess free RAP or mannose-6-phosphate were prepared.

Dilutions of RAP-GAA were made in the uptake medium (Dulbecco's Modified Eagle's Medium supplemented with 25 mM HEPES pH 7.0, 2 mM L-glutamine and 250 µg/mL bovine serum albumin) to yield fusion protein concentrations of 33, 11, 3.7, 1.2, 0.4, and 0.1 nM. The effect of 3 mM mannose-6-phosphate, 500 nM RAP and a combination of the two on the uptake of 5 nM RAP-GAA was also assayed. The GM244 fibroblasts were seeded into 12-well plates and allowed to grow for 3 days prior to the uptake experiment.

To initiate uptake, the growth medium was aspirated from the wells and each sample dispensed into duplicate wells at 1 ml per well. Plates were

incubated for 4 hours at 37°C, 5% CO₂. Samples were then aspirated from each well, the wells washed with phosphate-buffered saline (PBS), and pre-warmed 0.25% trypsin/ 0.1% EDTA added to each well at 37° for 5 minutes to release the adherent cells. Released cells were pelleted and rinsed with chilled PBS. Pre-chilled lysis
5 buffer (phosphate-citrate buffer, pH 4.0 with 0.15% Triton X-100) was then added and the pellets resuspended by gentle vortexing. Lysed cells could be stored at -80°C.

To measure the levels of GAA activity in the lysed cells, the frozen lysates were thawed at room temperature. Lysate (50 µl) was added directly to duplicate wells in 96-well opaque microtiter plates. Pre-warmed GAA fluorescent
10 substrate (4-methylumbelliferyl-alpha-D-glucoside, 100 µL) was added to each well to initiate the reaction. The plate was incubated at 37° C for 30 minutes and the reaction terminated by addition of 150 µl glycine/carbonate buffer pH 10. Fluorescence was measured in a plate reader at an excitation wavelength of 366 nm and an emission wavelength of 446 nm.

15 The results in Figure 13 show that RAP-GAA is taken up by GM244 fibroblast cells. The K_{uptake} was ~19 nM as determined by a non-linear fit enzymatic algorithm described in the GraFit software program (Sando and Neufeld, Cell, 12(3):619-27, 1977). Approximately 60-fold more RAP-GAA gets into the fibroblasts than recombinant GAA (V_{max} ratio); 25-fold more at 10 nM. Additionally,
20 90% of the RAP-GAA fusion uptake is inhibited by 50 nM RAP while only 20% of the uptake is inhibited by 3 mM mannose 6-phosphate. The uptake of the native GAA is almost completely inhibited by mannose 6-phosphate, suggesting alternate receptor pathways for RAP-GAA and recombinant GAA.

EXAMPLE 5

25 **Measurement of RAP-GAA uptake and lysosomal localization in LRPnull CHO cells expressing different LRP receptor family members (LRP1B, LDLR, VLDLR) and into BN cells expressing only LRP2 (Megalin, gp330).**

Iodine labeling: RAP-GAA or recombinant GAA were radiolabeled
30 with ¹²⁵I using the IODO-GEN reagent.

Cells were seeded in 12-well plates at a density of 200,000 cells/well and used after overnight culture. On the day of the experiment, cells were rinsed twice

in ice-cold ligand binding buffer (Minimal Eagle's medium containing 0.6% bovine serum albumin; BSA), and 125 I-RAP-GAA or GAA alone were then added in the same buffer (0.5 ml/well). The initial ligand concentrations tested were 10 nM. Binding was carried out at 4°C for 30 min with gentle rocking in the presence or absence of unlabeled 500 nM RAP or 10 mM mannose-6-phosphate to confirm receptor-binding specificity. Unbound ligand was then removed by washing cell monolayers three times with ice-cold binding buffer. Ice-cold stop/strip solution (0.2 M acetic acid, pH 2.6, 0.1 M NaCl) was then added to one set of plates without warming and kept on ice prior to counting. Dissociation constants for the receptor-ligand complexes were determined from the resulting binding data. The remaining plates were then placed in a 37°C water bath, and 0.5 ml of ligand binding buffer prewarmed to 37°C was added to the well monolayers to initiate internalization. At each time point (every 30 seconds for 2 minutes and every 3 minutes thereafter) the wells were placed on ice, and the ligand-binding buffer replaced with ice-cold stop/strip solution. Ligand that remained on the cell surface was stripped by incubation for 20 minutes (0.75 ml for 10 minutes, twice) and counted. Internalization rates were determined from this data. Cell monolayers were then solubilized with SDS lysis buffer (62.5 mM Tris-HCl, pH 6.8, 0.2% SDS, and 10% (v/v) glycerol) and counted. The sum of ligand that was internalized added to that which remained on the cell surface after each assay was used as the maximum potential internalization. The fraction of internalized ligand after each time point was calculated and plotted.

Measurement of ligand degradation efficiency (transport to lysosomes after internalization): Cells were seeded at a density of 200,000 cells/well into 12-well dishes 1 day prior to assays. On the day of the experiment, pre-warmed assay buffer containing RAP-GAA or GAA alone was added to cell monolayers in the presence or absence of unlabeled 500 nM RAP or 10 mM mannose 6-phosphate, followed by incubation for 4 hours at 37°C. Following incubation, the medium overlaying the cell monolayers was removed and proteins were precipitated by addition of BSA to 10 mg/ml and trichloroacetic acid to a final concentration of 20%. Lysosomal degradation of ligands was defined as the appearance of radioactive fragments in the medium that were soluble in 20% trichloroacetic acid. The protein concentrations of each cell lysate were measured in parallel dishes that did not contain LRP ligands. The RAP-GAA and GAA degradation efficiencies were calculated as the value of

degraded radioactive material (soluble cpm/mg cell protein) divided by the number of cell surface LRP family receptors (as determined previously by flow cytometry, data not shown).

EXAMPLE 6

5 **Measurement of specific uptake of RAP-LE in to enzyme-deficient patient fibroblasts with concomitant clearance of stored glycosaminoglycans**

Patient fibroblasts are seeded and grown to confluence in 12-well plates. On the day of the experiment, cells are fed with fresh medium lacking MgSO_4 and containing 4 $\mu\text{Ci/mL}$ of $\text{Na}_2^{35}\text{SO}_4$. Cells are also supplemented with RAP-LE
10 fusion or LE alone in the presence or absence of 500 nM RAP or 10 mM mannose 6-phosphate. Cells are harvested each day for 4 days. After rinsing with PBS, cells are lysed by freeze-thaw. Stored GAG is assayed by precipitation with 80% ethanol and quantitated by scintillation counting. Stored GAG values are normalized to the protein content of the cell lysates.

15 EXAMPLE 7

Measurement of lysosomal distribution and clearance of storage in intravenously-administered RAP-GAA in GAA-deficient mice

GAA knock out mice (C57Bl/6 background) were randomized to four treatment groups and treated every two days with 100 μl of either phosphate-buffered
20 saline, 1.3 mg/kg or 0.33 mg/kg RAP-GAA fusion protein four times via intravenous tail vein injection. Forty-eight hours after the fourth injection, mice were euthanized by carbon dioxide inhalation and the brain, heart, diaphragm, upper and lower body skeletal muscle and liver immediately collected and flash frozen. Three age-matched wild-type mice were also euthanized and tissues collected and frozen. Each tissue is
25 prepared for GAA immunohistochemical staining by embedding in OCT blocks, and for glycogen staining by fixing in glutaraldehyde and embedding in paraffin. The remaining tissues were tested for GAA activity using the fluorescent substrate assay described in Example 4. Serum was collected at sacrifice and tested for GAA antibody.

Dosing Regimen

Group	#Animals	Test Articles Or Vehicle Articles	Dose (mg/kg)	#Doses	Dose Volume (μl)
1	6 KO	PBS	-	4	100
2	6 KO	RAP-GAA	0.33	4	100
3	6 KO	RAP-GAA	1.30	4	100
4	6 KO	GAA	1.30	4	100
5	3 WT	None	None	None	None

Study day 0	Inject groups 1-4
Study day 2	Inject groups 1-4
Study day 4	Inject groups 1-4
Study day 7	Inject groups 1-4
Study day 9	Bleed groups 1-4 and Sacrifice groups 1-5, Collect tissues groups 1-5

5

EXAMPLE 8**Treatment of patients with MPS-I disorder**

A pharmaceutical composition comprising a conjugated agent comprising therapeutic enzyme linked to RAP is administered intravenously. The final dosage form of the fluid includes the conjugated agent, normal saline, phosphate
10 buffer at pH 5.8 and human albumin at 1 mg/ml. These are prepared in a bag of normal saline.

A preferred composition comprises the conjugated agent (therapeutic enzyme linked to RAP) in an amount ranging from 0.05-0.5 mg/mL or 12,500-50,000 units per mL; sodium chloride solution 150 mM; sodium phosphate buffer 10-50 mM,
15 pH 5.8; human albumin 1 mg/mL. The composition may be in an intravenous bag of 50 to 250 ml.

Human patients manifesting a clinical phenotype of deficiency of lysosomal enzyme, such as in patients with MPS I with an alpha-L-iduronidase level of less than 1% of normal in leukocytes and fibroblasts are included in the study. All patients manifest some clinical evidence of visceral and soft tissue accumulation of glycosaminoglycans with varying degrees of functional impairment. Efficacy is determined by measuring the percentage reduction in urinary GAG excretion over time. The urinary GAG levels in MPS-I patients are compared to normal excretion values. There is a wide range of urine GAG values in untreated MPS-I patients. A greater than 50% reduction in excretion of undegraded GAGs following therapy with the conjugated agent is a valid means to measure an individual's response to therapy. For example, data is collected measuring the leukocyte iduronidase activity and buccal iduronidase activity before and after therapy in MPS I patients. Clinical assessment of liver and spleen size is performed as it is the most widely accepted means for evaluating successful bone marrow transplant treatment in MPS-I patients (Hoogerbrugge et al., Lancet 345:1398, 1995).

EXAMPLE 9

Lysosomal storage diseases that may be treated with corresponding RAP-LE conjugates

The diseases that can be treated or prevented using the methods of the present invention are: Mucopolysaccharidosis I (MPS I), MPS II, MPS IIIA, MPS IIIB, Metachromatic Leukodystrophy (MLD), Krabbe, Pompe, Ceroid Lipofuscinosis, Tay-Sachs, Niemann-Pick A and B, and other lysosomal diseases. For each disease the conjugated agent would comprise a specific compound or enzyme. For methods involving MPS I, the preferred compound or enzyme is α -L-iduronidase. For methods involving MPS II, the preferred compound or enzyme is iduronate-2-sulfatase. For methods involving MPS IIIA, the preferred compound or enzyme is heparan N-sulfatase. For methods involving MPS IIIB, the preferred compound or enzyme is α -N-acetylglucosaminidase. For methods involving Metachromatic Leukodystrophy (MLD), the preferred compound or enzyme is arylsulfatase A. For methods involving Krabbe, the preferred compound or enzyme is galactosylceramidase. For methods involving Pompe, the preferred compound or enzyme is acid α -glucosidase. For methods involving CLN, the preferred compound or enzyme is tripeptidyl peptidase. For methods involving Tay-Sachs, the preferred compound or enzyme is

hexosaminidase alpha. For methods involving Niemann-Pick A and B the preferred compound or enzyme is acid sphingomyelinase.

EXAMPLE 10

Further Exemplification of receptor binding, cell uptake and lysosomal delivery of fusions of RAP and α -L-iduronidase or acid α glucosidase

The present example provides additional data demonstrating the efficient LRP receptor binding, cell uptake and lysosomal delivery of therapeutic enzymes using RAP as a delivery vehicle.

Fusion expression constructs-The human RAP coding sequence, encompassing amino acids 35-353, was amplified from human liver cDNA using PfuTurbo polymerase (Stratagene) and the primers RAPF 5'- G C G A T A G G A T C C T A C T C G C G G G A G A A G A A C C A G C C C A A G C C G T C C C C G A - 3' (SEQ ID NO:12) and RAPR 5' - G C G A T A A A C C G G T T T C T G C C T C G G C G C G A G C T C T G G A G A T C C T G C C G G A C A G G T C C T - 3' (SEQ ID NO:13). This fragment does not include sequence encoding either the signal peptide or the HNEL ER retention signal. The 5'-RAP primer incorporates an in-frame BamHI site at the 5' end. The 3'-RAP primer adds sequence encoding a six amino acid spacer (AEAETG; SEQ ID NO: 29) including an in-frame AgeI site at the 3'-end. The modified RAP sequence was cloned into the vector pC3B as an in-frame fusion with either human alpha-L-iduronidase (amino acids 27-652) or human alpha-glucosidase (amino acids 70-952). Both lysosomal enzyme sequences were 5'-modified to remove their signal peptides and to add an in-frame AgeI site. The expression vector is derived from pCDNA3.1 (+) (Invitrogen) and includes the rabbit beta-actin IVS2, the rat preproinsulin transcript leader sequence and the first 18 amino acids (signal peptide) of human melanotransferrin ending with an in-frame BamHI site.

Plasmid vectors were linearized with AclI and transfected into CHO-K1 LRP⁻ (CHOdL) using standard protocols. Clones were selected by limiting dilution in medium containing 800 μ g/mL G418. Clones were screened for expression using fluorescent monosaccharide substrates for the respective lysosomal



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Die folgenden Angaben sind den vom Anmelder eingereichten Unterlagen entnommen

54 Selektive proteolytische Synzyme (SPS)

57 Die Erfindung betrifft den Bereich Pharmakologie der
proteolytischen Substanzen bzw. Moleküle.

Die Aufgabe der Erfindung ist, kleine nicht Peptid ähnliche spezifische proteolytische Moleküle zu modellieren und zu erzeugen, die effektiv krankheitserregende Peptide und folglich Plaques spalten und Blut-Hirn-Schranke überwinden können.

Die Aufgabe der Erfindung wird dadurch gelöst, dass ein proteolytisches Enzym analog oder Synzym mit einem spezifischen Beta-Amyloid-(BA4), Prion (PrP^{Sc}) oder Huntingtin (Polyglutamin-Kette aus ungefähr 40-60 Glutaminen) oder einem anderen Molekül verbunden wird, damit ein Plaque oder ein krankheitserregendes (Poly)Peptid spezifisch gespalten werden kann. Dieses bifunktionale Molekül kann mit einem anderen Molekül wie z. B. einer Thiolgruppe, Fulleren oder mit einem Nitrit, Nitrat oder mit einem anderen vasodilatorischen Molekül u. a. Molekülen verbunden werden.

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Die Erfindung betrifft den Bereich Pharmakologie der proteolytischen Substanzen bzw. Molekülen.

Es gibt eine Strategie zur Behandlung der Krankheiten, die durch krankheitserregende Proteine verursacht werden. Eine Protease bzw. Protease-Domäne wird mit einem immunogenischen Peptid oder Antipeptid fusioniert, und somit wird die Substratspezifität dieser Protease nur auf bestimmte, durch das Antipeptid, erkennbare Proteine begrenzt.

Der Erfinder Alexander Cherkasky (s. Stern 25.3.1999 5.19) will die Substratspezifität einer Protease gegen Alzheimer-erregende Beta-Amyloide lenken, damit diese und folglich die von ihnen (Beta-Amyloiden) gebildete Plaques gespalten werden können. Da die proteolytischen Fusionsproteinen relativ groß sind, ist es fraglich, ob sie die Blut-Hirn-Schranke überwinden oder nicht.

Die Aufgabe der Erfindung ist kleine nicht Peptid ähnliche spezifische proteolytische Moleküle zu schaffen, die effektiv krankheitserregende Peptide und folglich Plaques spalten, und Blut-Hirn-Schranke überwinden können.

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In der Fig. 1 ist die allgemeine Struktur eines selektiven proteolytischen Synzyms schematisch dargestellt.

In der Fig. 1a ist eine Kombination aus einem proteolytischen Synzym 1 und einem spezifisch – bindenden Molekül 2 schematisch dargestellt.

In der Fig. 1b ist das proteolytische Synzym 1 zwischen der beiden spezifisch – bindenden Molekülen 2 schematisch dargestellt.

In der Fig. 1c ist das spezifisch – bindende Molekül 2 zwischen der beiden proteolytischen Synzymen 1 schematisch dargestellt.

In der Fig. 2 sind die Formeln einiger proteolytischen Synzyme dargestellt.

In der Fig. 2a ist die Formel eines Aspartyl – proteolytischen Synzyms dargestellt. Das ist ein Enzym analog von Aspartyl – proteasen.

In der Fig. 2b ist die Formel eines Serin – proteolytischen Synzyms dargestellt. Das Trizyklin ist das Basis-Molekül, an welchem die Aminosäurereste der katalytischen Asp-His-Ser-Triade von den Serinproteasen gebunden sind. Somit wird das aktive oder katalytische Zentrum von Serinproteasen imitiert.

In der Fig. 2c ist die Formel eines Cystein – proteolytischen Synzyms vereinfacht dargestellt.

Das Trizyklin ist auch das Basis-Molekül, an welchem die wichtigsten Cystein – und Histidin Aminosäureresten des Cystein – Proteasen gebunden sind. Ein selektives proteolytisches Synzym, welches in den Fig. 1 und 2 dargestellt ist, erkennt und bindet durch ein spezifisch – bindendes Molekül ein krankheitserregendes bzw. pathogenes (Poly) Peptid. Durch dieses spezifisch – bindendes Molekül wird das pathogene (Poly) Peptid durch das proteolytische Synzym selektiv bzw. spezifisch gespalten.

Nach der Spaltung wird das pathogene (Poly) Peptid wie z. B. Beta-Amyloid, Huntingtin, Prion – Protein, u.v.a. Proteine nicht mehr gefährlich, weil die extrazellulären Plaques

gespalten und danach abgeführt werden. Die Klasse von selektiven proteolytischen Wirkstoffen wird vom Erfinder als "Proteopharmaka" oder "Proteolytica" bzw. "Proteolytica selectiva" bezeichnet.

Patentansprüche

1. Selektive Proteolytische Synzyme (SPS), **dadurch gekennzeichnet**, dass ein proteolytisches Synzym ein Basis-Molekül enthält, an welchem proteolytische Aminosäurereste wie z. B. Asp-His-Ser oder Asp-Asp gebunden sind
2. Selektive Proteolytische Synzyme (SPS), dadurch gekennzeichnet, dass ein proteolytisches Enzym analog (Synzym) mit einem spezifischen Beta-Amyloid (BA4), Prion (PrP^{Sc}), oder Huntingtin (Polyglutamin) – bindenden oder einem anderen Molekül verbunden wird. Durch das entstandene Molekül wird ein Plaque oder ein pathogenisches bzw. krankheitserregendes (Poly) Peptid spezifisch bzw. selektiv gespalten.
3. Selektive Proteolytische Synzyme (SPS), dadurch gekennzeichnet, dass das Molekül nach den Ansprüchen 1 und 2 mit einem Antioxidant, wie z. B. ein Schwefel – oder Selenium haltendes Molekül, oder ein Fulleren, einem Vasodilator, wie z. B. ein Nitrit, Nitrat oder ein Phosphodiesterase-Inhibitor, oder einem anderen Molekül verbunden wird.
4. Selektive Proteolytische Synzyme (SPS), dadurch gekennzeichnet, dass die Klasse von selektiven proteolytischen Wirkstoffen vom Erfinder als "Proteopharmaka" oder "Proteolytica" bzw. "Proteolytica selectiva" bezeichnet wird.

Hierzu 2 Seite(n) Zeichnungen

Fig. 1

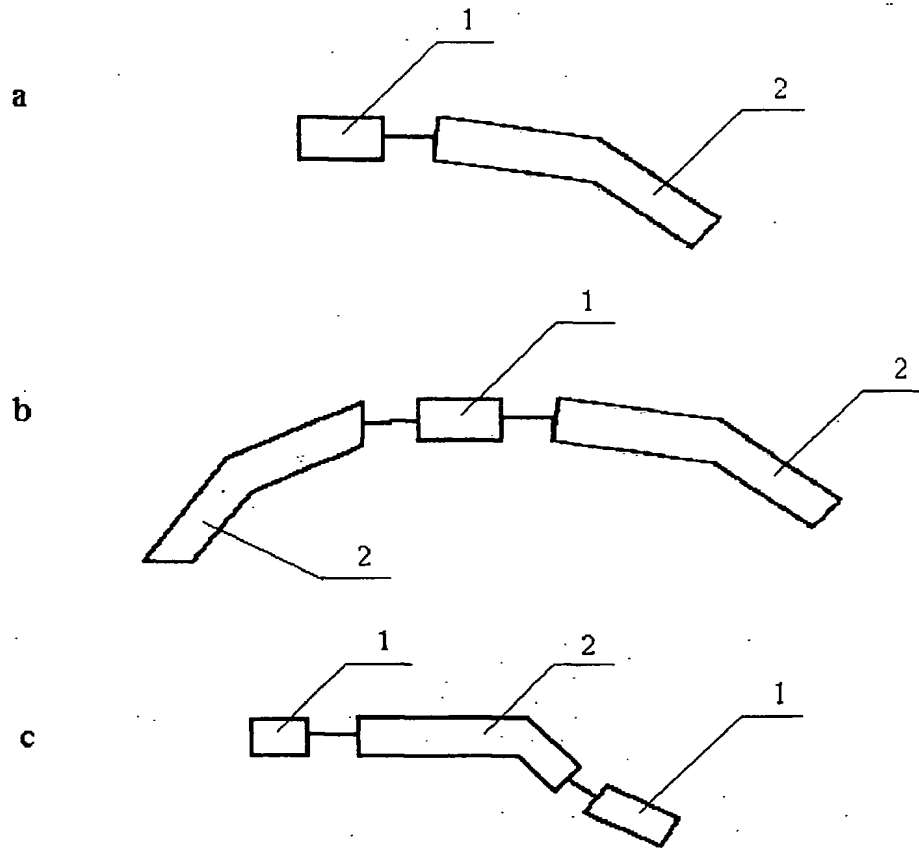
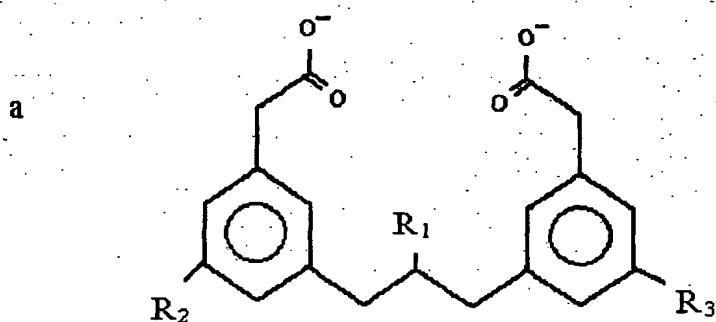
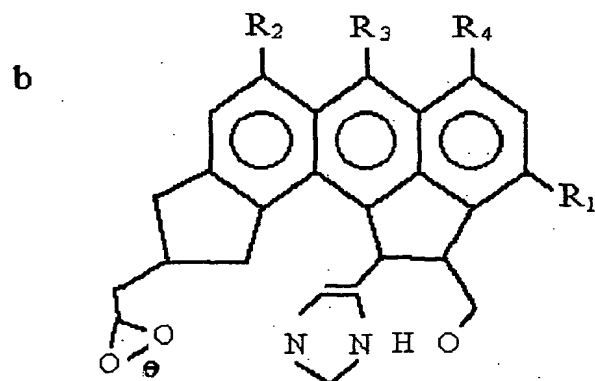


Fig. 2



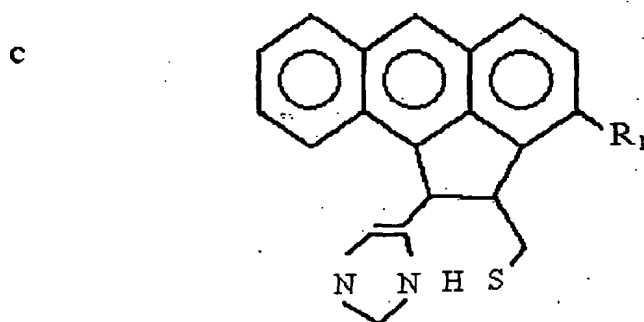
R_1 : - H, - CH₃, - OH, = 0

$R_2 = R_3$: spezifische bindende Moleküle



R_1 : spezifisches bindendes Molekül

$R_2 = R_3 = R_4$: - OH (hydrophil), - SH (antioxidativ)



R_1 : spezifisches bindendes Molekül



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(54) Title: METHOD OF ASSAY OF INHIBIN		
(57) Abstract A method of immunoassay for the estimation of inhibin in an inhibin-containing sample which comprises the step of using an antibody directed against inhibin. Preferably, the antibody is contained in an antiserum raised by injecting an animal with an antigen selected from the group consisting of naturally-occurring or recombinant inhibin, or sub-units, fragments or derivatives thereof. The assay may suitably be a radioimmunoassay, a fluorescence-based immunoassay, or an enzyme-linked immunosorbent assay using labelled 58kD or 31kD inhibin as tracer. Tracers and standards for use in the assay are described and claimed.		

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- 1 -

METHOD OF ASSAY OF INHIBIN

5 This invention relates to methods for assay of inhibin, and in particular to methods for immunoassay of inhibin.

Background Art

Two forms of inhibin from bovine follicular fluid
10 have been recently purified to homogeneity, with molecular masses of 58kD and 31kD (International Patent Application PCT/AU/85/00119 and Robertson et al 1985, 1986). Under reducing conditions both forms consist of two subunits with molecular masses of 43kD and 15kD, and 20kD and 15kD
15 respectively. Their primary amino acid structures have been

elucidated following cloning and analysis of cDNA species derived from bovine granulosa cell mRNA (International Patent Application PCT/AU86/00097; Forage et al 1986). These studies indicate that 31kD inhibin is a processed form of the 58kD molecule. 31-32kD inhibin molecules with similar subunit structures to bFF inhibin have been isolated from porcine follicular fluid (Miyamoto et al 1985, Ling et al 1985) and sequenced (Mason et al 1985).

Currently inhibin activity is measured by a variety of in vivo and in vitro bioassay systems (Baker et al 1981). These systems are time consuming, expensive, have limited sensitivity and precision and are of limited practicability in their application to large sample numbers (Baker et al 1981, Lee et al 1982).

15 Summary of the Invention

The present invention relates to more convenient assays for the estimation of inhibin than have heretofore been possible. The preferred assays of the invention are radioimmunoassays and the following description, whilst being directed to the preferred assays, should not be construed as limiting the invention to radioimmunoassays. Other assays within the scope of the invention include ELISAs, immunoassays based on fluorescence detection, and related assays relying on polyclonal and monoclonal antibodies against inhibin.

25 Preferred Embodiments of the Invention

According to one aspect of the present invention there is provided an immunoassay for the estimation of inhibin in an inhibin-containing sample which comprises the step of using an antibody directed against inhibin.

30 Preferably the antibody is contained in an antiserum raised by injecting an animal with an antigen selected from the group consisting of naturally-occurring or recombinant inhibin, or sub-units, fragments or derivatives thereof. Particularly preferred antigens include preparations containing 35 inhibin, purified bovine 58kD inhibin,

purified bovine 31kD inhibin, human inhibin, or human or bovine inhibin or fragments thereof produced using recombinant DNA technology.

Suitable animals include mammals such as mice, rabbits, horses, donkeys, dogs, sheep, and goats, and birds such as chickens.

Alternatively a monoclonal antibody or an IgG directed against any of the aforesaid inhibins may be used.

Most preferably the antibody is capable of neutralizing inhibin bioactivity.

Preferably the immunoassay is further characterized by the step of using labelled 58kD or 31kD inhibin as tracer. More preferably said tracer is labelled with ^{125}I iodine (^{125}I) with an enzyme, or with a fluorescent marker.

Preferably the assay is a radioimmunoassay or an enzyme-linked immunosorbent assay (ELISA), or a fluorescence-based immunoassay.

15 The invention provides a method for measuring inhibin in samples such as follicular fluid or serum from various species (including humans) wherein concentrations of inhibin in standards are used to derive the concentration of inhibin in the follicular fluid or serum by competitive
20 binding of ^{125}I labelled inhibin and inhibin from test samples with bovine 58kD inhibin antiserum, followed by precipitation and counting of bound ^{125}I labelled inhibin.

 The preferred specific radioimmunoassay system for inhibin of the invention is applicable to bovine and human
25 follicular fluid and serum, and can employ an antiserum against (purified bovine) 58kD inhibin with iodinated 31kD or 58kD inhibin as tracer.

 According to a second aspect of the invention, there is provided a method for preparation and purification of
30 ^{125}I -labelled inhibin tracer which comprises the steps of iodination of inhibin using a Chloramine T procedure and purification of ^{125}I -inhibin by an affinity fractionation step.

 Preferably the affinity fractionation step uses
35 Matrex Red A.

 Preferably the purification procedure additionally comprises a gel filtration step.

- 4 -

According to a third aspect of the invention there is provided an assay standard selected from the group consisting of naturally-occurring or recombinant inhibin, or fragments or derivatives thereof. Preferably the standard displays parallelism in the assay with the samples under test.

Particularly preferred standards include bovine 31kD inhibin, and partially purified or purified human inhibin.

The conditions of the assay, in particular incubation times, may be varied in order to attain desired levels of sensitivity.

A form of the radioimmunoassay modified for increased sensitivity comprises:

incubating sample and antiserum for 4 days at 4°C, followed by the addition of ^{125}I -31kD inhibin tracer, incubating for 3 days at 4°C and then adding second antibody, precipitating, and counting bound ^{125}I labelled inhibin.

According to one particularly preferred embodiment, suitable for measurement of inhibin in human serum samples, the tracer is ^{125}I -31kD inhibin, and incubation with tracer is performed at elevated temperature (30°C) in the presence of inhibin-free serum, in order to minimize non-specific effects. Suitable sources of inhibin-free serum include steers or other castrated male animals, oophorectomized women, women with premature ovarian failure, and post-menopausal women.

25 Brief Description of the Drawings

Figure 1 shows the fractionation of ^{125}I -58kD and ^{125}I -31kD inhibin on analytical SDS-PAGE under reducing conditions.

Figure 2 shows the time course of immunization of a rabbit with 58kD inhibin.

Figure 3 shows the in vitro neutralization of bFF inhibin by an antiserum raised to 58kD inhibin.

Figure 4 shows the radioimmunoassay dose response curves of bFF, hFF, purified 58kD and 31kD inhibin and bovine granulosa cell culture medium (BGCM) using either ^{125}I -31kD or ^{125}I -58kD inhibin as tracers.

- 5 -

Figure 5 shows the profile of inhibin in vitro bioactivity and immunoactivity following fractionation of bFF through the various steps of the inhibin purification procedure of Robertson et al (1986).

5 Figure 6 shows non-reduced SDS-PAGE profiles of ^{125}I -58kD and ^{125}I -31kD inhibin following incubation with bFF and serum under conditions used in the RIA of serum inhibin.

Figure 7 shows the effect of temperature on the binding of ^{125}I -31kD inhibin to the antiserum.

10 Figure 8 shows logit-log dose response lines of bovine and human serum, in the plasma RIA system employing ^{125}I -31kD inhibin as tracer.

Figure 9 shows the ovulation induction regime and serum levels of FSH, LH, inhibin and oestradiol (E_2) in 15 twenty-six women involved in an In Vitro Fertilisation (IVF) programme and one normal woman (FL#27).

Figure 10 is a comparison of plasma E_2 and inhibin levels plotted for some of the data in Figure 9.

Figure 11 shows the correlation between the number 20 of ova produced and E_2 or inhibin levels in serum.

Figure 12 shows the correlation between the numbers of ovarian follicles detected ultrasonically and peak inhibin levels in serum.

Figure 13 shows inhibin, FSH, progesterone and 25 oestradiol concentrations in the sera of non-pregnant subjects ($n = 16$) on the days following oocyte retrieval.

Figure 14 shows inhibin, FSH, progesterone and oestradiol concentrations in the sera of pregnant subjects ($n = 3$) on the days following oocyte retrieval.

30 Figure 15 shows the relationship between serum inhibin and FSH during the luteal phase of non-pregnant subjects. For this analysis, non-detectable inhibin values ($n = 29$) were assigned the limit of assay sensitivity.

Figure 16 shows inhibin, FSH, LH, oestradiol and progesterone concentrations in the sera of normal women during the menstrual cycle, assayed using anti-31kD inhibin.

- 6 -

Detailed Description of the InventionAbbreviations

	bFF	:	bovine follicular fluid
	hFF	:	human follicular fluid
5	oFF	:	ovine follicular fluid
	oRTF	:	ovine rete testis fluid
	HPLC	:	high performance liquid chromatography
	SDS-PAGE:		sodium dodecyl sulphate polyacrylamide gel electrophoresis
10	RIA	:	radioimmunoassay
	SS	:	steer serum
	BS	:	bull serum
	CS	:	cow serum
	PMS	:	human post-menopausal serum
15	HFP	:	human female plasma

Preparations(a) Purification of bFF inhibin

The purification of bFF 31kD and 58kD inhibin was based on the procedures described previously (Robertson et al 20 1985, 1986)(Figure 5):

(a) bFF was fractionated on a Sephacryl S200 (9 x 90 cm) gel filtration column in 0.05M ammonium acetate pH 7.0.

(b) The void volume fraction from (a) was precipitated at pH 4.75 and fractionated on Sephadex G100 (9 x 90 25 cm) in 4M acetic acid.

(c) and (d)

Peak I (58kD inhibin) and Peak II (31kD inhibin) fractions from (b) were fractionated on an RPSC 30 Ultrapore column (0.46 x 7.6 cm, Beckman) using a 0-50% acetonitrile gradient in 0.1% trifluoroacetic acid. In Figure 5 the continuous line indicates optical density at 280 nm (a) and (b) and 254 nm (c) and (d).

35 Hatched area denotes inhibin bioactivity.
o---o RIA with ¹²⁵I-58kD inhibin as tracer.

- 7 -

o__o RIA with ^{125}I -31kD inhibin as tracer.

V_o = void volume.

BSA = bovine serum albumin (mol. wt 67,000).

OVA = ovalbumin (mol. wt 43,000).

5 The purified inhibin was stored in SDS
electroelution buffer (approx. 3% SDS in 10 mM NH_4HCO_3) prior
to iodination. For bioassay, samples were methanol
precipitated at -20°C in order to remove SDS and solubilized
by heating at 37°C for 1 hour and sonication.

10 Similar profiles of both bio- and immunoactive
inhibin were observed at each stage of the inhibin
purification procedure. The biological to immunological
activity ratios for a number of purified 31kD and 58kD inhibin
preparations using both tracers in the radioimmunoassay ranged
15 from 0.30 - 0.43.

(b) Sample Preparation

Human follicular fluid was obtained at oocyte
collection in the in vitro fertilisation programme at the
Queen Victoria Medical Centre/Epworth Hospital, Melbourne. It
20 was charcoal treated (100 mg/ml dextran-coated charcoal for 1
hour at 4°C), lyophilised, stored at -20°C and resolubilized
prior to assay by sonication in assay buffer or culture
medium. Ovine follicular fluid (oFF) was obtained by
aspiration of ovaries collected at a local abattoir. Ovine
25 rete testis fluid (oRTF) is a lyophilised inhibin preparation
(Baker et al 1985). Rat ovarian extract was a
charcoal-treated rat ovarian cytosol preparation. Details of
the biopotencies of these inhibin preparations are outlined in
Table 1.

30 Blood was collected in lithium heparin tubes from 40
women undergoing ovulation induction therapy (clomiphene
citrate and human menopausal gonadotrophin treatment) with
plasma estradiol levels at time of plasma collection ranging
from 40-2,900 pg/ml. Equal aliquots of serum from each
35 subject were combined to produce a plasma pool designated as

- 8 -

Human Female Plasma pool (HFP). Plasma from four post-menopausal women aged 52 and over were combined to give a pool designated Post-Menopausal Serum pool (PMS).

Bovine blood, ovaries and testes were collected on ice from a local abattoir and processed within one hour. All samples were stored at -20°C after snap freezing in solid CO_2 /ethanol. Blood pools from adult intact (BS, $n = 9$) and castrate (SS, $n = 1$) male, and female (CS, $n = 10$) cattle were allowed to clot overnight at 4°C prior to centrifugation and storage. Bovine ovarian follicles were hemisected and granulosa cells were collected by aspiration and cultured for 40 hours at a concentration of 10^5 viable cells per well (Costar 48 well plate) in $400\ \mu\text{l}$ DMEM/F12 complete medium. Media were stored frozen at -20°C prior to assay. Testes from four bulls were decapsulated and homogenised in equal w/v Dulbecco's phosphate buffer using an Ultra-Turrax tissue disperser (Janke and Kunkal KS, Staufen FRG) and centrifuged at $100,000\text{g} \times 1\text{ hour}$ at 4°C and stored at -20°C . Prior to assay the supernatants were charcoal treated with an equal volume of 1% Norit A in Dulbecco's phosphate buffer and incubated at 4°C for 30 minutes prior to centrifugation and bioassay (Au et al 1983).

Immunization Procedure

Purified 58kD inhibin ($14\ \mu\text{g}$ in $500\ \mu\text{l}$ Dulbecco's phosphate buffer) was emulsified in an equal volume of adjuvant (Marcol 52 [Esso, Australia]: Montanide 888 [S.E.P.P.I.C., Paris] in the ratio 9:1) and injected into an intact male New Zealand white rabbit 4 intramuscular and one subcutaneous sites. Two booster injections of $14\ \mu\text{g}$ under the same conditions were given at six weeks and one year. Serum was collected throughout for assessment of its in vitro neutralization activity, its ability to bind iodinated inhibin and for plasma FSH estimations.

A similar procedure was used to raise antibody directed against purified 31kD inhibin.

Analytical SDS Polyacrylamide Gel Electrophoresis

Sera and bFF were incubated at various temperatures in an equal volume of 100 mM phosphate buffer pH 7.4 containing 0.15M NaCl, 0.1% Triton X-100 and either 0.5% BSA for studies with ^{125}I -31kD inhibin or 0.5% Polypep for ^{125}I -58kD inhibin. Equal volumes (5 μ l) of sample and 10% SDS and Dulbecco's Phosphate buffer pH 7.4 (30 μ l) were placed in a boiling water bath for 2.0 minutes then in ice. Ten microlitres of bromophenol blue (0.006%) in glycerol (62.5% in H_2O) was then added and the mixture centrifuged prior to electrophoresis on 12.5% slab gels (3 hours, 20-30 mA). Protein molecular weight markers were either co-electrophoresed with the iodinated sample in the absence of bFF and serum or on a separate track in their presence. The gels were fixed and stained overnight in ethanol: H_2O formaldehyde (180: 420: 100) containing 0.1% Coomassie Brilliant Blue. Each track was divided into 50 2mm slices and counted in a gamma counter.

Reversed-phase HPLC

^{125}I -Inhibin was applied to an Ultrapore RPSC column (0.46 x 7.5 cm, Beckman, Berkeley, Ca., USA) and fractionated using a 30 min linear gradient of 0.50% acetonitrile in 0.1% trifluoroacetic acid at 1 ml/min and 0.5 ml fractions using Waters HPLC apparatus (model 6000A pumps and a model 660 Programmer, Milford, Mass., USA).

In Vitro Bioassay

Inhibin activity was determined using an in vitro bioassay based upon the dose-dependent suppression of FSH cell content in rat pituitary cell cultures utilizing a parallel line bioassay design (Scott et al 1980). The charcoal-treated bovine follicular fluid preparation employed a lymph reference preparation with an arbitrary unitage of 1 unit/mg (Scott et al 1980).

Hormone Assays

- 10 -

(i) Rabbit FSH Radioimmunoassay

Rabbit FSH was determined using an RIA kit kindly provided by Dr. A.F. Parlow (Torrance, Ca, USA) employing 15% polyethylene glycol to separate bound and free hormone. The sensitivity of the assay was 0.9 ng/ml using rabbit FSH AFP.538.C as standard. The within assay coefficient of variation was 8.1% and all samples were assayed in the one assay.

(ii) Rat FSH Radioimmunoassay

Rat FSH generated by the pituitary cells in culture was measured by a specific radioimmunoassay using reagents supplied by the NIAMDD. ^{125}I -rat FSH (I_5) was used as tracer and FSH RP-2 used as standard. The within-assay coefficient of variation was 7%.

(iii) RIA of human hormones

Serum FSH was measured by RIA (Amerlex-M, Amersham, USA) using 2nd IRP FSH as standard with an interassay CV of 7.0% from 31 assays. LH was measured by RIA (LH RIA, Diagnostic Products Corp., L.A., USA) using the 2nd IRP LH as standard with an interassay CV of 10.1% from 31 assays. Both oestradiol and progesterone were measured using RIA (Coat-a-Count, Diagnostic Products Corp., L.A.) with interassay CVs of 8.7% and 8.1% respectively from 150 assays. Serum beta subunit of hCG was measured by RIA (B-hCG RIA-Quant, Mallinckrodt Inc. St. Louis, USA) using the hCG 2nd IS as standard with an interassay CV of 10.4% from 30 assays.

Calculations

The RIA dose-response curves were linearised using a logit-log dose transformation. Parallelism was assessed from a comparison of slope values of dose-response curves using the multiple range test for groups of unequal size (Kramer, 1956) or by paired t-test. Potency estimates were determined using standard parallel line bioassay statistics. In situations where non-parallelism was observed between dose response lines

- 11 -

of unknown and standard preparations, potency estimates were determined from the ratio of their ED_{50} values. The sensitivity (ED_{10}) was defined as the mass of hormone required to give 10% displacement in the assay whilst ED_{50} corresponded to the mass required for 50% displacement.

The index of precision (Gaddum (1933); Finney (1964)) was used to describe assay precision. The between assay variation was calculated from the coefficient of variation of the repeated measurement of a partially purified inhibin preparation. The dissociation constant (K_{dis}) was determined by Scatchard analysis using ^{125}I -hormone and increasing amounts of unlabelled hormone. The mass of ^{125}I -hormone used in the analysis was determined from its specific activity ($\mu Ci/\mu g$).

The invention will now be illustrated by reference to the following non-limiting examples.

Example 1 Antiserum Characterization

Antisera against 58kD and 31kD inhibin were characterized by showing that following immunization, parallel changes in plasma FSH and inhibin antibody titre were observed, indicating inhibin neutralization in vivo. The antisera neutralized bFF, hFF and purified 31kD and 58kD inhibin activity in an in vitro bioassay. The results described below refer to anti-58kD inhibin, but similar results were obtained using anti-31kD inhibin.

(a) Response In Vitro to Immunization

Following the first booster (Fig. 2a) the antibody titre was assessed by the ability of the antibody to neutralize inhibin bioactivity in vitro and its capacity to bind ^{125}I -58kD inhibin. A sharp parallel elevation in these activities was observed between 1-8 weeks post-booster injection. During this period significant ($P < 0.05$) elevation in serum FSH (6.63 ± 0.95 ng/ml, $n = 5$ vs 4.97 ± 0.87 ng/ml, $n = 6$ (mean \pm 1 SD)) was noted. Following the second booster (Fig. 2b), an immediate and sustained elevation of serum ^{125}I -31kD inhibin-binding capacity and serum FSH was observed.

- 12 -

Basal levels of serum FSH were assessed from the mean + 2 S.D. of 14 observations over the preceeding five months (hatched area Figure 2b).

These results indicate that purified inhibin from 5 bovine follicular fluid can be used to immunize rabbits, producing an antiserum which has the capability of neutralizing inhibin bioactivity. The elevated levels of plasma FSH in the rabbit observed during the period of peak antiserum titre (as assessed by iodinated inhibin binding and 10 in vitro neutralizing capacity) indicate that the antibodies produced are capable of neutralizing endogenous inhibin. The combination of the neutralization of inhibin both in vivo and in vitro and the close relationship of the neutralizing activity and iodinated inhibin binding capacity of the 15 antiserum provides convincing evidence of its specificity.

(b) Inhibin Neutralisation In Vitro

Bovine follicular fluid inhibin (2 units) was quantitatively neutralised by 1 μ l antiserum per culture well while 75% inhibition of bioactivity was achieved with 0.35 μ l 20 per well (Fig. 3, Table 1). In Figure 3, the vertical dotted line indicates the volume of antiserum required to achieve 75% neutralization of inhibin activity, an arbitrary parameter of antiserum neutralizing titre. Purified 58 and 31kD inhibin gave corresponding 75% inhibition values of 0.33 and 0.38 μ l 25 respectively. In comparison, neutralisation of hFF inhibin bioactivity required 1.32 μ l antiserum (n = 2, Table 1) corresponding to 27% cross-reactivity in comparison to bFF inhibin. Inhibin from ovine sources (follicular fluid, rete testis fluid) showed 8 and 6% cross-reactivity respectively. 30 This antiserum, at a maximum non-toxic level of 4 μ l per well, did not neutralise 2 units of inhibin activity in rat ovarian cytosol extracts.

TABLE 1
Cross-reactivity of inhibin from various sources as assessed by inhibin neutralization in vitro and radioimmunoassay

IN VITRO NEUTRALIZATION

125 I-58kD Inhibin

125 I-31kD Inhibin

Preparation	Inhibin Bioactivity U/ml	Antiserum Titre (μ l)	% Cross- Reactivity	ED ₅₀ (U)	% Cross- Reaction	ED ₅₀ (U)	% Cross- Reaction
<u>Follicular Fluid</u>							
Bovine	5200	0.35 \pm 0.04 (6)	100	1.30 \pm 0.25 (9)	100	0.99 (2)	100
Human	168 \pm 32 (6)	1.32 (2)	27	5.3 \pm 1.6 (4)	28.4 \pm 8.7 (4)	2.7 (2)	37
Ovine	27,000 (2)	4.4 (2)	8.0	> 320	< 0.3	> 320	< 0.1
<u>Ovarian Extract</u>							
Rat	384 (2)	> 6 (2)	< 6.0	**		**	
<u>Rete Testis Fluid</u>							
Ovine	1040*	> 6.0 (1)	< 6.0	> 52	< 2	> 52	< 2

* U/mg protein

** No displacement with 16 U inhibin
Mean \pm SD (n)

- 14 -

Example 2 Iodination of inhibin

Iodination of either 58kD or 31kD inhibin has been achieved using a conventional Chloramine T iodination procedure and was associated with considerable iodination damage. Purification of the tracer was therefore necessary and it was not achieved following gel filtration chromatography on Sephadex G25. Specific binding of either iodinated hormone to Matrex Red A achieved satisfactory purification although recoveries were low. Either iodinated inhibin form thus purified had the physico-chemical properties of its non-iodinated form.

Alternative iodination procedures using Iodogen, Iodobeads, lactoperoxidase, or Bolton-Hunter reagent were found to cause less damage to the inhibin molecule, but resulted in poorer incorporation of radioactivity. Consequently iodination using Chloramine T was preferred.

Purified 58kD or 31kD inhibin (1-2 µg in 25 µl electroelution buffer was added to 25 µl 0.5 M phosphate buffer, pH 7.2. Na¹²⁵I (0.5 mCi, 5 µl; Amersham, Bucks, UK) was added. Chloramine T (40 µl) was added at a ratio of 8:1 Chloramine T to hormone. The reaction proceeded for 60 seconds at room temperature with stirring and was terminated with 20 µl sodium metabisulphite (3 mg/ml). The reaction mixture was made up to 50 µl in 20mM phosphate buffer 0.1% BSA or 0.5% Polypep (Sigma, St. Louis, Mo., USA) pH 6.0 and gel filtered on a Sephadex G25 column (PD10, Pharmacia, Uppsala, Sweden) to remove ¹²⁵I. The void volume fractions were pooled, made up to 20 ml and applied to a column of 200 µl Matrex Red A (Amicon, Danvers, Mass., USA) and then washed with phosphate buffer containing 400 mM KCl, the eluted counts being discarded. ¹²⁵I-inhibin was eluted with 1M KCl/4M urea in phosphate buffer. The iodinated inhibin was further gel filtered on a Sephadex G25 column (PD10) with the appropriate RIA buffer (see below) to remove the KCl/urea.

Following iodination of 58kD and 31kD inhibin, 60 pCi and 25 pCi respectively were recovered in the void volume fractions following gel chromatography on Sephadex G25.

Approximately 30% was eluted with the 1M KCl/4M urea buffer. ^{125}I -inhibin, as assessed by its molecular weight on SDS-PAGE, was found in this fraction.

The specific activity of the iodinated preparations was assessed in the radioimmunoassay using a self-displacement procedure (Marana et al 1979) with the hormone used for iodination as standard. Specific activities of 50-60 $\mu\text{Ci}/\mu\text{g}$ for 58kD inhibin and 24 $\mu\text{Ci}/\mu\text{g}$ for 31kD inhibin were obtained, with recoveries ranging from 5-25%.

10 Example 3 Characterisation of iodinated inhibin

The physico-chemical characteristics of ^{125}I -inhibin were assessed using RP-HPLC and SDS-PAGE. A close correspondence was observed between the radioactive and bioactive profiles on RP-HPLC for both the 58kD and 31kD preparations (data not shown). The molecular mass of ^{125}I -58kD inhibin following fractionation on SDS-PAGE was similar to purified non-iodinated inhibin under both non-reducing (58kD) and reducing (43kD and 15kD) conditions except that a 58kD material of unknown identity was observed in relatively low proportions (18%) under reducing conditions (Fig. 1). The molecular weight markers employed were BSA (bovine serum albumin) 67,000; OV (ovalbumin) 43,000; CA (carbonic anhydrase) 29,000; GL (goose egg lysozyme) 20,300; and CL (chick egg lysozyme) 14,300. The arrow, in figure 1, refers to the point of sample application. Radioactivity found in fractions beyond fraction 47 represents free iodine in the solvent front. The purity of the inhibin used for iodination as assessed by silver staining on SDS-PAGE suggests that the ^{125}I -58kD material is not an iodinated contaminant. In support, ^{125}I -58kD inhibin was fractionated by microelectrofocusing procedure on the pH range 3.5-10 and 4-8 (Foulds and Robertson 1983), and 3 peaks of radioactivity with pI values of 7.4, 6.2 and 5.2 were observed. Upon reduction on SDS-PAGE each of these peaks showed persistence of ^{125}I -58kD material. The results suggest that the presence of

- 16 -

the ^{125}I -58kD material is attributable to difficulties in reduction of the iodinated hormone rather than to the iodination of a contaminating protein.

Fractionation of the ^{125}I -31kD inhibin on SDS-PAGE revealed molecular weights of 30,200 under non-reducing conditions and 20,000 and 15,000 subunits following reduction; these values are similar to those for the non-iodinated hormone. A second antibody RIA system using either tracer yielded a parallel displacement between purified 31kD and 58kD inhibin.

Example 4 Radioimmunoassay Procedure

The assay buffer used was 10 mM phosphate, 0.15 M NaCl, 0.5% BSA, pH 7.2. A delayed tracer addition, second antibody assay system was employed. The sample and antiserum were incubated in a volume of 300 μl for 16 hours at room temperature following which ^{125}I -inhibin (10,000 cpm, 100 μl) was added and the incubation continued either overnight at room temperature or for 48 hours at 4°C. Second antibody (goat antiserum to rabbit IgG, 100 μl) was added and incubated for 1 hour at 4°C following which 1 ml 6% polyethylene glycol was added. The tubes were vortexed and incubated for a further 30 min, spun at 2000 g for 30 min at 4°C, decanted and counted. The inclusion of Triton X-100 (final concentration 0.025%) in the assay buffer reduced non-specific binding from 4 to 0.5%.

Radioimmunoassay procedures were established using both 31 and 58kD inhibin tracers. Following a logit-log dose transformation of the response curves, linear displacement of each tracer was observed for a range of inhibin preparations, with the exception of 31kD inhibin when using ^{125}I -58kD inhibin as tracer, in which a deviation from linearity below logit -0.5 (38% B/B₀) was seen (Fig. 4). In figure 4, each value represents the mean \pm SD of triplicates. The characteristics of each assay are outlined in Table 2. Scatchard analysis revealed similar affinities for the antiserum of either inhibin form. Non-parallel dose response

- 17 -

lines were observed between bFF and either 31kD inhibin with ^{125}I -31kD inhibin as tracer of 58kD inhibin with ^{125}I -58kD inhibin as inhibin tracer. The sensitivity (ED_{10}) and ED_{50} values were comparable in each assay with either hormone.

- 18 -

TABLE 2

Characteristics of the two radioimmunoassay systems with
 ^{125}I -31kD inhibin and ^{125}I -58kD inhibin as tracers

	^{125}I -31kD inhibin	^{125}I -58kD inhibin
5		
Antibody Dilution	1:8000	1:4000
Tracer binding (Bo)	30%	18%
Affinity (Kdis) $\times 10^{-10}\text{M}$ 20°C	0.66	0.72
10 ED ₁₀ (ng, fm)		
31kD inhibin	0.10, 3.0	0.13, 4.4
58kD inhibin	0.07, 1.2	0.13, 2.2
ED ₅₀ (ng)		
31kD inhibin	0.30, 10.1	0.51, 17.1
15 58kD inhibin	0.26, 4.3	0.43, 7.0
Slope*		
bFF	1.37 \pm 0.09 ^a (8)	1.47 \pm 0.09 ^c (8)
31kD inhibin	1.53 \pm 0.09 ^b (5)	1.68 \pm 0.08 (3)
58kD inhibin	1.50 \pm 0.07 (3)	1.73 \pm 0.14 ^d (5)
20 Precision**	0.036 (5)	0.038 (5)
Between Assay Variation**	14 (5)	8.5% (5)
Bio/Imm Ratio		
31kD inhibin	0.34 \pm 0.09 (16)	0.43 \pm 0.13 (4)
58kD inhibin	0.30 \pm 0.12 (7)	0.37 \pm 0.12 (5)
25 BGCM	0.25 (1)	-

a vs b P < 0.05)

) as assessed by paired t-test

a vs c P < 0.01)

30 Mean \pm SD

* Number in brackets: number of preparations

** Number in brackets: number of assays

BGCM = bovine granulosa cell culture medium

For details see text.

- 19 -

Example 5 Specificity of the assay

The specificity was assessed on the following grounds. First, a similar hierarchy of cross-reaction of inhibin from various species in the RIA using either tracer 5 and in vitro neutralisation studies was observed (Table 1). The cross-reaction in the radioimmunoassay of inhibin from different species when expressed in terms of their bioactivity was bFF 100%, hFF 30% ovine FF 1% and rat ovarian extract non-detectable. With respect to this antiserum it is apparent 10 that both male and female bovine and human inhibin share common antigenic determinants not found in inhibin from the other two species. This implies close structural similarity between inhibin from both sexes and species. Secondly, no cross-reaction (0.5%) occurred for a range of purified 15 glycoproteins and polypeptides. Rat LH and FSH, ovine LH and FSH, hCG, bovine TSH, LHRH, ovalbumin and bovine serum albumin showed less than 0.5% cross reactivity using either tracer. Alternatively, medium from the bovine granulosa cell culture (Fig. 4), and bovine testis extract (data not shown), both 20 containing inhibin bioactivity, gave parallel displacement curves to bFF inhibin in the RIA. The parallel dilution of inhibin bio- and immunoactivity of medium from bovine granulosa cell culture with the inhibin standard provides evidence for these cells being the site of inhibin production, 25 as has been previously suggested (Erickson and Hsueh 1978; Henderson and Franchimont 1981). Thirdly, similarities were observed in the profiles of both biological and immunological activities following fractionation of bFF on gel filtration chromatography and RP-HPLC. However, in the 40-60kD molecular 30 mass region of the Sephacryl S200 column (Figure 5a), an 8-40 fold excess of immunoactivity over bioactivity was present, accounting for 12-18% of the recovered immunoactivity.

A large variation in the ratio of biological/immunological activities with charcoal-treated bFF 35 as standard was observed following fractionation of bFF inhibin on gel filtration and RP-HPLC (Fig. 5) and between purified inhibin preparations (Table 2). The ratios ranged

- 20 -

from 0.02-2.09 in fractions obtained during the purification procedure and from 0.30-0.43 with the purified inhibin preparations.

It is concluded that the RIA procedures are not
5 detecting molecular entities devoid of biological activity and
vice versa except in the lower molecular weight region
(40-60kD) of the Sephacryl S200 chromatogram. Whether this
lower molecular weight material represents a protein distinct
from inhibin which cross-reacts in the RIA or inhibin devoid
10 of biological activity has not been established.

The cross-reactivities of inhibin-related proteins
in the RIA relative to 31 kDa bFF inhibin were as follows:
porcine transforming growth factor-beta (R & D Systems, Minn.
USA) <0.9%, bovine Mullerian inhibitory substance (kindly
15 provided by Dr. J. Hutson, Royal Children's Hospital,
Melbourne) <0.3% purified bovine inhibin B subunit dimer <2%
and the subunits of 31 kDa bFF inhibin obtained following
reduction and alkylation <0.1%. A range of glycoproteins and
growth factors have been previously tested (McLachlan et al,
20 1986) and showed cross reactivities against anti-58kD inhibin
of less than 1.0%. The specificity of anti-31kD inhibin was
similar, with cross-reactivities of less than 1.0%.

Example 6 Application of the Radioimmunoassay to Serum

The RIA in its application to serum required
substantial modification. Firstly 100mM phosphate buffer pH
7.4 containing 0.15M NaCl, 0.5% BSA was used, and, because of
25 its stability in serum, ^{125}I -31kD inhibin was preferred to
 ^{125}I -58kD inhibin as RIA tracer. Secondly, a
temperature-dependent interference of steer serum with
 ^{125}I -31kD inhibin binding to the antiserum was observed, with
an increase in binding (B/Bo) from 57% at 4°C to 94% at 37°C
30 (Fig. 7). This figure demonstrates the temperature dependence
following a 16 hour incubation in the presence of various
serum and inhibin preparations (bFF, 31kD inhibin, steer serum
(SS), cow serum (CS), bull serum (BS), human post-menopausal
serum (PMS), human female serum pool (NFP)). Tracer binding
35 (B/T) was maximal at 30°C in the presence of steer serum,
being $87.1 \pm 3.4\%$ (n = 5). Displacement of ^{125}I -31kD inhibin

- 21 -

by bFF or 31kD inhibin was largely unaffected by temperature. Human PMS showed no temperature related interference upon binding although the binding was elevated (B/Bo 110-120%).

Based on these data, conditions for the assay of either bovine or human serum inhibin were established. These involved using ^{125}I -31kD inhibin as tracer in an overnight 30°C incubation and, in order to compensate for the low level of interference by SS or PMS (presumed to contain no inhibin) standards and samples were diluted in SS or PMS accordingly.

No detectable activity was determined in steer or in human post-menopausal serum, whilst bull and human female serum showed parallel dose-response curves to their respective follicular fluid standards, with circulating levels of 0.9 and 1.1 ng respectively.

Inhibin preparations or serum samples were diluted in SS or PMS to a sample volume of 200 µl. Antiserum (100 µl, final dilution 1:8000) and samples (200 µl) were incubated for 4 hours at 30°C, followed by a further incubation of 16 hours at 30°C in the presence of tracer. Second antibody was added and the tubes were incubated for 24 hours at 4°C, following which 2 ml 0.15M NaCl was added and the tubes were centrifuged.

Thirdly, with respect to a choice of standard in the RIA of bovine serum inhibin, purified 31kD inhibin is favoured in view of its stability in serum. In the absence of a purified human inhibin preparation, 31kD bovine inhibin may be used as the standard in the RIA of human serum inhibin. However, the partially purified hFF inhibin preparation described above is preferred, and purified hFF inhibin, when available, would be the most preferred standard. The detectable levels of inhibin immunoactivity in serum from women under going ovarian stimulation with exogenous gonadotrophin is analogous to the findings of Lee et al (1982), where circulating levels of inhibin activity were detected in PMSG treated immature female rats, particularly directed against 58kD and 31kD inhibin.

- 21a -

Individual antisera may behave differently in the assay, and assay parameters may have to be determined for each case. Considerable variations in sensitivity between antisera have been observed, particularly between antisera directed against 31kD and 58kD inhibin. Anti-31kD inhibin appeared to give greater sensitivity than anti-58kD inhibin in the samples tested so far.

- 22 -

Example 7 Improved Sensitivity RIA for human serum

In order to improve assay sensitivity, the assay procedure above was modified as follows: the total volume of the assay was reduced from 400 to 300 μ l (comprising 200 μ l sample, 50 μ l tracer and 50 μ l antiserum). The assay buffer was 150 mM phosphate, 0.2% BSA pH 7.4, and the incubation of sample and antiserum was 4 days at 4°C followed by the addition of tracer and a further 3 days at 4°C prior to the addition of second antibody. Using this method, a 2.5 fold increase in sensitivity was achieved. This modified assay procedure has been applied to the measurement of human plasma inhibin. The modified assay allows the quantification of plasma inhibin in normal male plasma and in plasma throughout the normal menstrual cycle.

15 Example 8 Stability of 125 I-31kD and 125 I-58kD Inhibin in Serum

SDS-PAGE profiles of 125 I-58kD inhibin following overnight incubation with serum (SS and PMS) showed an increased formation of an 125 I-30kD component (12% of recovered activity at 4°C; 17% at 30°C) in comparison with either buffer or bFF (6% 4°C and 30°C; (Fig. 6). The tracers were incubated overnight at either 4 or 30°C with either bFF, steer serum (SS) or human post-menopausal serum (PMS). Incubation of either tracer in RIA buffer alone gave similar profiles to the bFF incubation shown. Molecular weight markers are described in Figure 1. No radioactivity was observed between the position of the marker carbonic anhydrase and the solvent front with either tracer. Results are presented as the mean \pm SD of three replicate experiments. In contrast, SDS-PAGE profiles of 125 I-31kD inhibin under the same incubation conditions showed no significant changes. Recoveries of radioactivity with either tracer were not affected by either temperature or by the presence of bFF or serum.

- 23 -

Example 9 Radioimmunoassay of inhibin in bovine serum

The application of the inhibin RIA to serum from cattle resulted in parallel logit-log dose response lines of BS with either bFF or 3lkD inhibin as standards (Fig. 8). The response shown in figure 8 is for bovine and human serum, diluted in steer serum or post-menopausal serum respectively, in the plasma RIA system employing ^{125}I 3lkD inhibin as tracer. Potential RIA standards (bFF, 3lkD inhibin, hFF) were assayed in the presence of either phosphate buffer (200 μl , o) or 10 steer serum (200 μl , ●) or post-menopausal serum (200 μl , ▲) and their logit plots were calculated using their respective non-specific binding and B_0 values. Cow serum shows a minimal detectable immunological response. When the immunoactivity was expressed in terms of 3lkD inhibin standard the level of 15 inhibin in BS was 0.91 ± 0.27 ($n = 3$) mg/ml and CS 0.1 ng/ml. A parallel response line of HFP was observed in the RIA with 3lkD inhibin and hFF as standards corresponding to levels of 1.05 ± 0.07 ($n = 3$) ng/ml. The levels in normal plasma ($n = 8$) were equal to or less than the sensitivity of the assay 20 (0.1 ng/ml).

Example 10 Radioimmunoassay of inhibin in infertile human subjects

The radioimmunoassay previously described was applied to plasma and serum from post-menopausal subjects 25 (presumed to be inhibin free, $n = 8$) and young women with ovarian failure (premature ovarian failure $n = 2$, Turner's syndrome $n = 1$, ovariectomized $n = 1$). There was no difference in ^{125}I -3lkD inhibin binding in the assay between these two groups and therefore post-menopausal serum was used 30 as a diluent in the assay.

Example 11 Radioimmunoassay of inhibin in human serum

The method was applied to:

(a) Plasma from normal women during spontaneous menstrual cycles from the early follicular phase until the 35 time of ovulation ($n = 2$, example 'FL' #27, Fig. 9). Inhibin

- 24 -

immunoactivity was below the limit of assay detection prior to day 13 and its increase correlated with the increase in circulating levels of oestradiol (E_2), LH and FSH on day 13 and 14.

5 (b) Inhibin immunoactivity in serum from normal men ($n = 7$) was at the lower limit of assay detection.

(c) Plasma was obtained from 26 unselected women undergoing their fourth cycle of ovulation induction in the in vitro fertilization programme at the Epworth Hospital, 10 Richmond, Victoria. Briefly this involved the administration of clomiphene citrate 100-150 mg daily on day 5-9 of the menstrual cycle, followed by human menopausal gonadotrophin (hMG) 75-225U daily for the next 5-7 days. Adequate follicular development was assessed by the progressive 15 increase in plasma oestradiol and by ovarian ultrasound. Ovulation occurred spontaneously if an endogenous LH spike was observed, or in the absence of the latter, ovulation was induced by administration of 5000 IU of human chorionic gonadotrophin (hCG). For an example of a spontaneous 20 ovulation see 'BU' # 11, Fig. 9k and for an hCG-induced ovulation sample see 'JO' 1, Fig. 9a.

Inhibin immunoactivity in the plasma samples, as defined in terms of the biological activity of a purified 31kD inhibin standard, showed a highly significant correlation with 25 plasma oestradiol levels (Fig. 10). The correlation coefficient values have been calculated from the total data in figure 9. An example of the correspondence of plasma oestradiol and inhibin during an ovulation induction cycle is seen in example 'BE' # 9, Fig. 9b. There was also significant 30 correlation between peak plasma oestradiol concentrations and the number of oocytes recovered, and peak plasma inhibin concentrations and the number of oocytes recovered (Fig. 11), as well as a strong correlation between the peak plasma inhibin concentration and the number of ovarian follicles 35 detected ultrasonically prior to oocyte aspiration (Fig. 12). It is therefore apparent that both plasma inhibin and plasma

- 25 -

oestradiol are parameters of follicular development and health, and in the majority of cases these show a close correspondence.

In certain examples ('BY' # 6, 'BR' # 10, Fig. 9h and j), a dissociation between plasma inhibin and plasma oestradiol concentrations was observed, suggesting different regulation of these two parameters of follicular development. As inhibin is a peptide produced by ovarian granulosa cells and plasma oestradiol in the human is predominantly an ovarian theca cell product, the assay of plasma inhibin is the first direct plasma parameter of granulosa cell/oocyte health and maturation. The dissociation of plasma inhibin and E_2 may therefore be of therapeutic importance in that the plasma inhibin is a direct measure of follicular development, and its assessment may affect the timing of ovulation induction and oocyte collection.

Example 12 hFF inhibin as standard for radioimmunoassay

Human follicular fluid (hFF) obtained at oocyte collection in the IVF programme was prepared for use as the radioimmunoassay (RIA) standard by two gel chromatographic steps and reversed phase HPLC as described for bFF inhibin (Robertson et al, 1985). This material yielded parallel dose response lines to human female serum inhibin obtained from women undergoing ovarian hyperstimulation for in vitro fertilisation. This partially purified human follicular fluid (hFF) inhibin standard preparation was defined in terms of its in vitro inhibin bioactivity using an inhibin bioassay based on the dose-related suppression of FSH cell content (Scott et al, 1980). This material was used as the RIA standard and gave dose response lines parallel to serum inhibin obtained from women undergoing ovarian hyperstimulation for in vitro fertilization and also to that of pregnant serum.

The unitage of the hFF inhibin standard was calibrated in terms of an ovine testicular lymph standard preparation of defined unitage 1 U/mg using the inhibin

- 26 -

bioassay. The RIA has an interassay coefficient of variation (CV) of 6.4% (n = 5 assay) and the sensitivity (logit + 2) was 0.37 U/ml.

The RIA was specific to bovine and human inhibin and cross-reacted less than 0.3% with a range of glycoproteins and growth factors. In addition, inhibin-related peptides cross-reacted as follows: porcine transforming growth factor β < 0.9%, bovine Mullerian Inhibitory Substance 0.3%, purified bovine inhibin B subunit dimer <1% and the subunits of 31kDa bFF inhibin following reduction and alkylation <0.1%. No immunoactivity was detectable in the sera of castrate subjects, post-menopausal women, nor in a subject with Turner's syndrome. The RIA had an interassay coefficient of variation of 8.3% (n = 5 assays), and a sensitivity of 0.37 U/ml.

Inhibin levels were determined at 1 dilution level against the partially purified hFF standard preparation using an iterative curve-fitting procedure (Burger et al., 1973). In the calculation of results, a lognormal distribution of individual observations (Gaddum et al; 1933) was assumed, i.e. all calculations were performed using logarithmically transformed values to give geometric means and 67% confidence intervals. Statistical comparison between pregnant and non-pregnant groups was performed using the unpaired t-test.

25 Example 13 Inhibin levels during luteal phase and early pregnancy

Nineteen women presenting consecutively for treatment in the Mondsh university IVF program were studied. Clinically their infertility resulted from tubal disease (n = 7), endometriosis (n = 6), unknown causes (n = 5) or poor semen quality (n = 4). The protocol of ovulation induction has been described elsewhere (Wood and Trounson, 1984). Briefly, all subjects received clomiphene citrate (Clomid, Merrell Dow, Sydney) 100-150 mg daily between days 5 and 9 of the cycle and HMG (Pergonal, Serono, Rome) 75-225 units daily from day 6. The dosage and duration of HMG therapy were optimized

- 27 -

according to daily plasma oestradiol concentrations and follicular size as assessed by ovarian ultrasound. HCG (Pregnyl, Organon, Oss) 5,000 IU intramuscularly was administered to induce ovulation, and oocyte retrieval was undertaken 36 hours later. Embryo transfer was performed as described by Wood and Trounson (1984). Blood was taken on day 1 post laparoscopy and every second day from day 2 to day 14 and sera stored for measurement of FSH, LH, β subunit hCG, oestradiol, progesterone and inhibin. Three of the 19 women became pregnant.

At various stages of gestation, a single serum sample was obtained from each of 24 normal pregnant women.

Samples were assayed for inhibin using the hFF inhibin standard described in Example 11. In the 16 patients who did not conceive, luteal phase inhibin levels rose to a peak level of 2.5 U/ml on day 6 and then fell to undetectable levels by day 14. These results are shown in Figure 13.

The number of subject serum samples per day was 13-16 except at day one when only eight were available. Results are expressed as the geometric mean \pm 67% confidence intervals. The broken line indicates the limit of sensitivity of the inhibin radioimmunoassay. The number of subjects showing non-detectable inhibin values is shown in parentheses. Non-detectable values are not included in the mean \pm confidence intervals.

In three subjects who conceived, inhibin levels were similar to non-conception cycles between days 2 and 8, increasing thereafter and becoming significantly higher ($p < 0.001$) than in the non-pregnant group by day 12 post-laparoscopy. Figure 14 shows these results, expressed as the geometric mean \pm 67% confidence intervals. The broken line indicates the sensitivity of the inhibin radioimmunoassay. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ comparing hormone values for the pregnant and non-pregnant groups on the same day. Significance values in the second panel refer to serum FSH. The late luteal phase rise in serum inhibin in the

- 28 -

pregnant patients coincided with both the rise in serum Δ hCG and with the decline in serum FSH to values below those seen in the non-pregnant group.

Serum FSH showed a significant inverse correlation with inhibin in the luteal phase of the non-conception cycles ($r = 0.51$, $n = 113$, $p < 0.001$) (Fig. 15). Similar significant inverse relationships were observed between FSH and inhibin when the data were analysed according to whether the progesterone concentrations were in the normal ovulatory luteal phase range (25-100) nm or greater, ($r = 0.38$, $n = 76$, $p < 0.001$ vs $r = 0.37$, $n = 37$, $p < 0.05$, slopes not statistically different). Significant inverse correlations also existed between luteal phase serum FSH and both progesterone ($r = 0.64$, $n = 113$, $p < 0.001$) and oestradiol ($r = 0.52$, $n = 114$, $p < 0.001$).

Plasma inhibin and progesterone concentrations were significantly correlated in the luteal phase of cycles in which pregnancy did not occur ($r = 0.81$, $n = 85$, $p < 0.001$), as were plasma inhibin and oestradiol concentrations ($r = 0.65$, $n = 85$, $p < 0.001$). In pregnant subjects, luteal phase inhibin levels did not show significant correlations with either progesterone or oestradiol. Serum LH levels (not shown) fell sharply from day 1 (21.0 [17.0-26.1] mIU/ml) to a nadir (3.5 [1.2-9.8] mIU/ml) on day 8.

In a separate study of serum inhibin, levels were determined during gestation in 24 normal pregnant women. The mean level prior to 20 weeks gestation (1.31 (0.95-1.80) U/ml, $n = 13$) was significantly lower ($p < 0.02$) than levels after this time (2.02 U/ml (1.32-3.10) U/ml, $n = 11$).

Thus there is a rise in circulating inhibin concentrations during the luteal phase of stimulated menstrual cycles and during pregnancy.

Example 4 Inhibin Levels in the Normal Menstrual Cycle

In a further study, serum inhibin was determined daily in 6 normal women throughout the menstrual cycle, using a radioimmunoassay employing an antiserum directed to 31kD inhibin. The normalcy of the menstrual cycle was assessed from the serum profiles of FSH, LH, progesterone, and oestradiol. The increase in sensitivity of the assay using this antiserum permitted the detection of inhibin in over 97% of samples. The results are shown in Fig. 16.

- 30 -

10 Advantages and applications of the assay according to the
invention.

1. The assay may be used for determining inhibin concentration in a wide range of biological samples, such as serum, plasma, urine, follicular fluid, tissue homogenates,
15 and culture fluids.
2. The assay may be used to monitor the purification of inhibin from tissue, biological fluids, or culture medium, or to monitor transfection studies.
3. Inhibin levels may be used as a marker of parameters of reproductive function, such as granulosa cell function, follicular development, number of ovarian follicles following ovarian hyperstimulation, and foetal well-being during early pregnancy, and Sertoli cell function.

It will be clearly understood that the invention in
25 its general aspects is not limited to the specific details referred to hereinabove.

The following terms referred to hereinbefore are trade marks: Amerlex-M, Clomid, Coat-a-Count, Marcol 5 2, Matrex Red A, Montanide 888, Norit A, Pergonal, Polypep,
30 Pregnyl, RIA-Quant, Sephacryl, Sephadex, Triton X-100, Ultrapore, and Ultra-Turrax.

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CLAIMS:

1. A method of immunoassay for the estimation of inhibin in an inhibin-containing sample which comprises the step of using an antibody directed against inhibin.
2. A method according to claim 1 in which the antibody is contained in an antiserum raised by injecting an animal with an antigen selected from the group consisting of naturally-occurring or recombinant inhibin, or sub-units, fragments or derivatives thereof.
3. A method according to claim 2 in which the antigen is selected from the group consisting of preparations containing inhibin, purified bovine 58kD inhibin, purified bovine 31kD inhibin, human inhibin, or human or bovine inhibin or sub-units, fragments or derivatives thereof produced using recombinant DNA technology.
4. A method according to claim 1 in which the antibody is a monoclonal antibody.
5. A method according to claim 1 in which the antibody is capable of neutralizing inhibin bioactivity.
6. A method according to claim 1 which further comprises the step of using labelled 58kD or 31kD inhibin as tracer.
7. A method according to claim 1 in which the assay is a radioimmunoassay, an enzyme-linked immunosorbent assay, or an immunoassay based on fluorescence detection.
8. A method according to claim 1 in which an assay standard is used, said assay standard being selected from the group consisting of naturally-occurring or recombinant inhibin, or sub-units, fragments or derivatives thereof.
9. A method according to claim 8 in which the standard displays parallelism in the assay with the samples under test.

10. A method according to claim 8 in which the standard is selected from the group consisting of bovine 31kD inhibin, partially purified human inhibin, and purified human inhibin.
11. A radioimmunoassay for measuring inhibin in a biological sample, comprising the steps of:
- a) incubating sample and antiserum for 4 hours to 4 days at 4° to 30°C,
 - b) adding ^{125}I -inhibin and incubating either overnight at room temperature, for 48 to 72 hours at 4°C, or for 16 hours at 30°C,
 - c) Adding a second antibody and incubating for 30 min. to 24 hours at 4°C,
 - d) Separating precipitate, and
 - e) counting bound ^{125}I -labelled inhibin.
12. A radioimmunoassay according to claim 11 in which samples to be assayed are diluted in inhibin-free serum.
13. A radioimmunoassay according to claim 11 in which an assay standard is used, said standard being selected from the group consisting of naturally-occurring or recombinant bovine 31kD inhibin and naturally-occurring or recombinant human inhibin.
14. A radioimmunoassay according to claim 11 in which incubation with ^{125}I -inhibin is at 30°C.
15. A radioimmunoassay according to claim 11 in which polyethylene glycol is added following incubation with the second antibody and incubated for a further 30 minutes.
16. A radioimmunoassay according to claim 11 in which Triton X-100 is incorporated into samples to be assayed.
17. A method for measuring inhibin in samples such as follicular fluid or serum from various species (including humans) wherein concentrations of inhibin in standards are used to derive the concentration of inhibin in the follicular

fluid or serum by competitive binding of ^{125}I -labelled inhibin and inhibin from test samples with bovine 58kD inhibin antiserum, followed by precipitation and counting of bound ^{125}I -labelled inhibin.

18. A method for preparation and purification of ^{125}I -labelled inhibin tracer which comprises the steps of iodination of inhibin using a Chloramine T procedure and purification of ^{125}I -inhibin by an affinity fractionation step.

19. A method according to claim 18 in which the affinity fractionation step uses Matrex Red A.

20. A method according to claim 18 which additionally comprises a gel filtration step.

21. An assay standard for estimation of inhibin according to the method defined in claim 1, claim 11 or claim 17, selected from the group consisting of naturally-occurring or recombinant inhibin, or fragments or derivatives thereof.

22. An assay standard according to claim 21 selected from 31kD bovine inhibin and human inhibin.

23. A test kit for the estimation of inhibin in a sample, comprising an agent selected from the group consisting of:

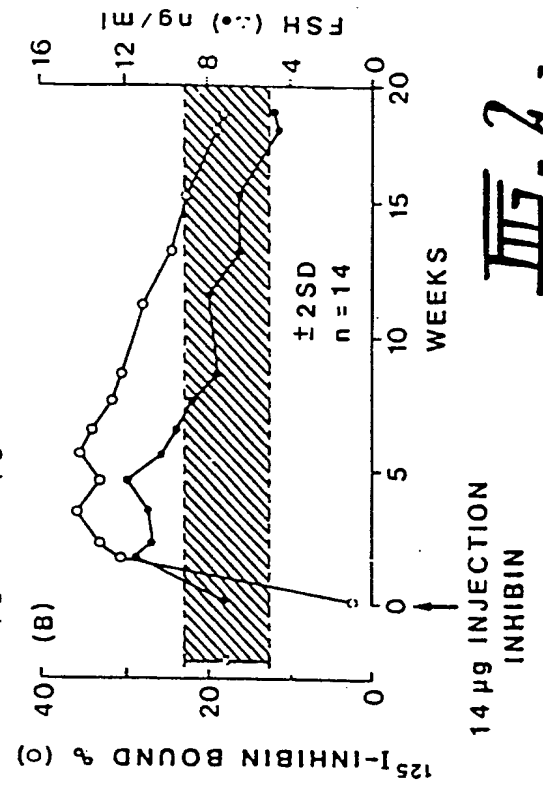
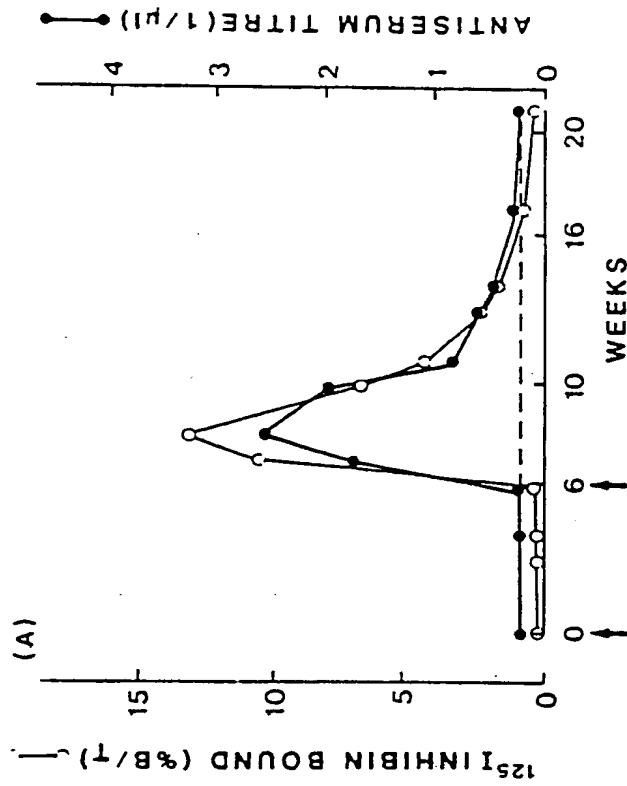
- a) labelled inhibin,
- b) antibody directed against inhibin,
- c) an assay standard as defined in claim 21.

24. A test kit according to claim 23 in which labelled inhibin is prepared according to the method of claim 18.

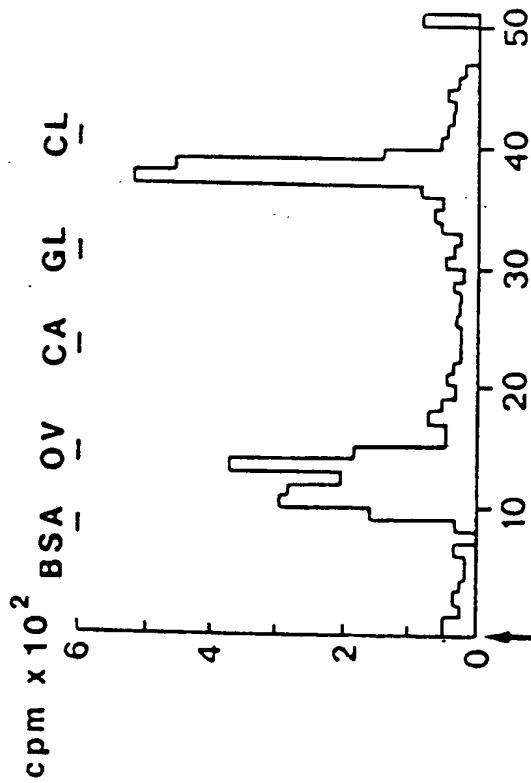
25. A test kit according to claim 23 in which the antibody to inhibin is contained in an antiserum as defined in claim 2.

26. Products and processes substantially as hereinbefore described with reference to the accompanying drawings.

1/20



58KD INHIBIN REDUCED



31KD INHIBIN REDUCED

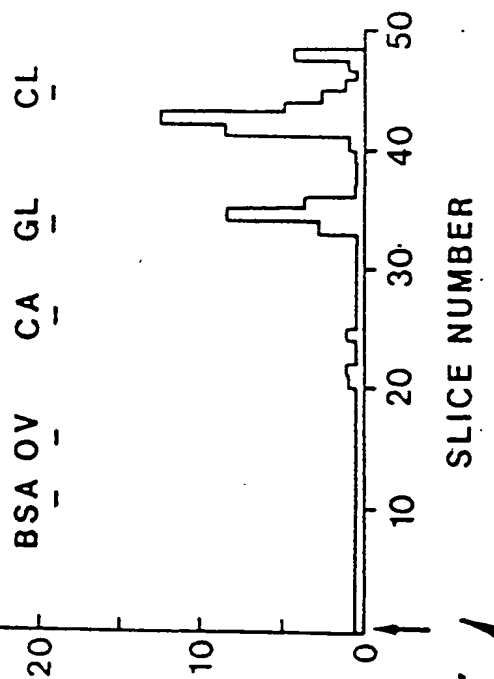
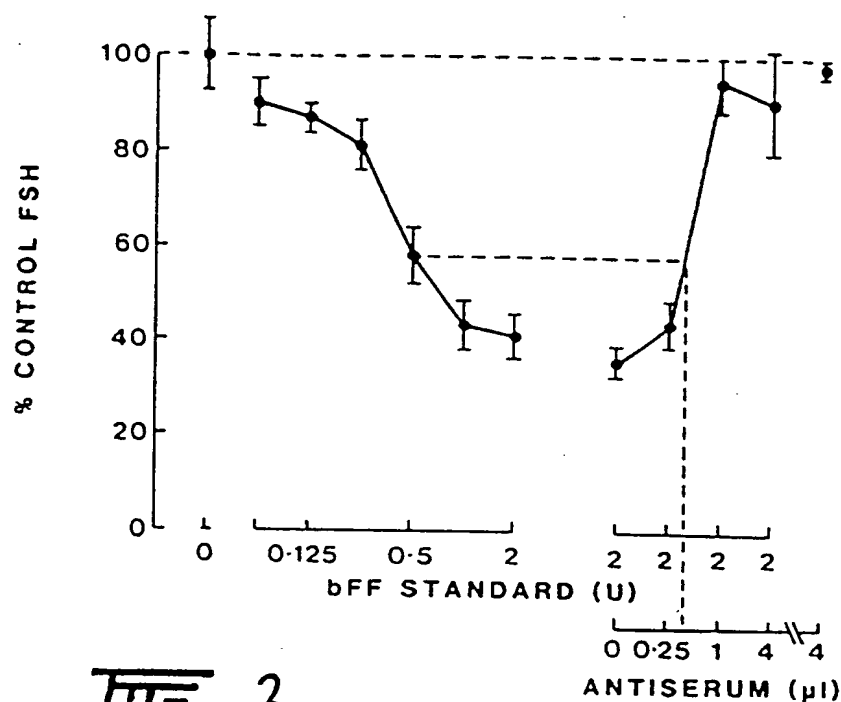


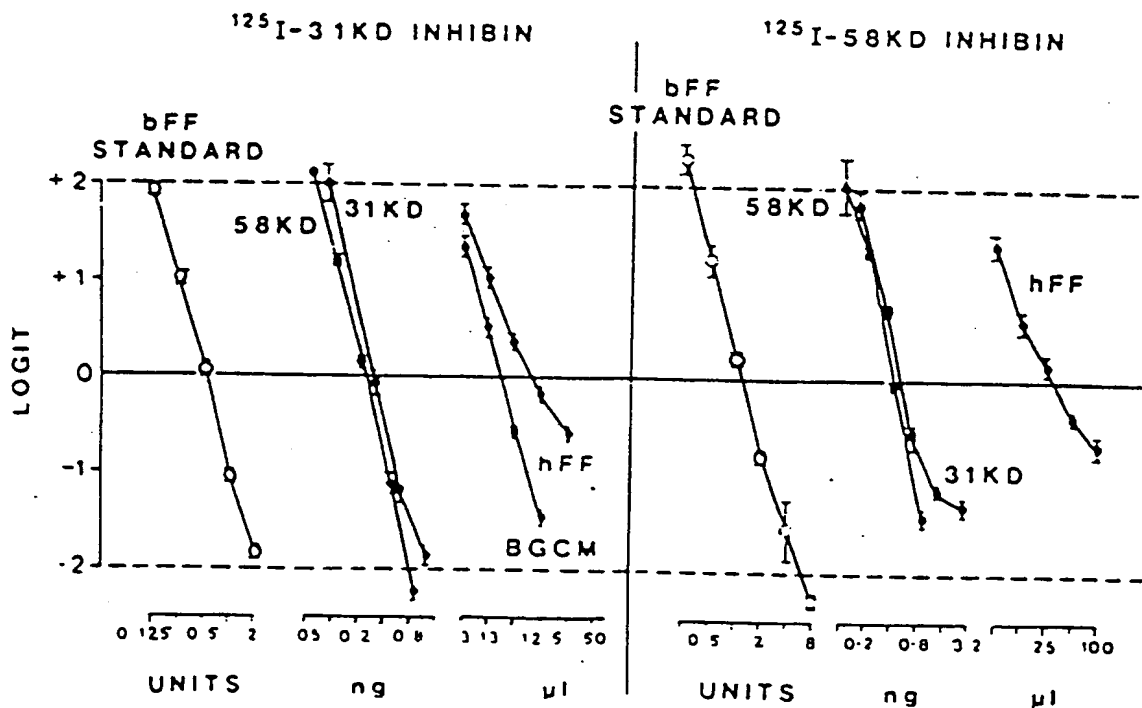
Fig. 2.

Fig. 1.

2/20

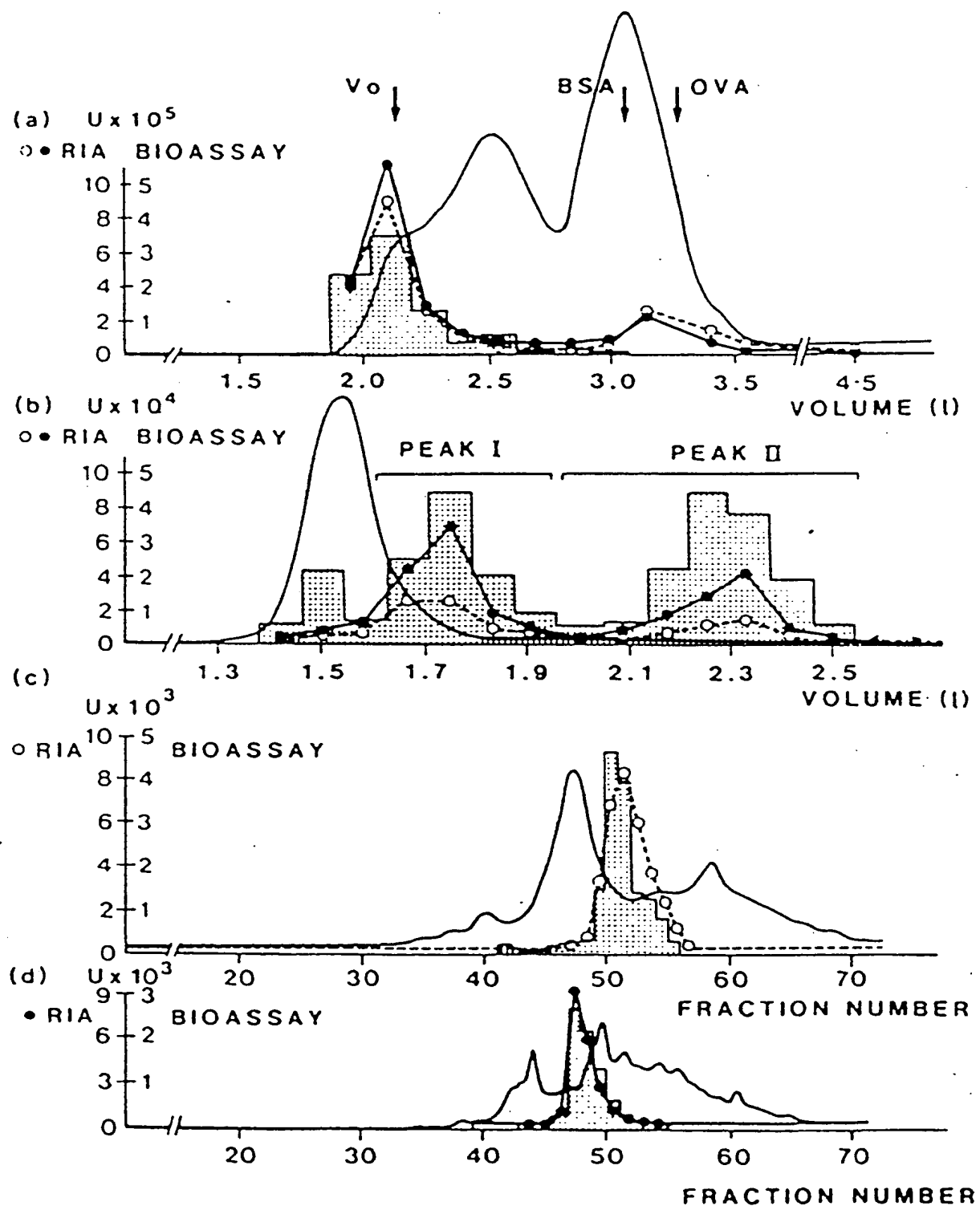


III. 3.



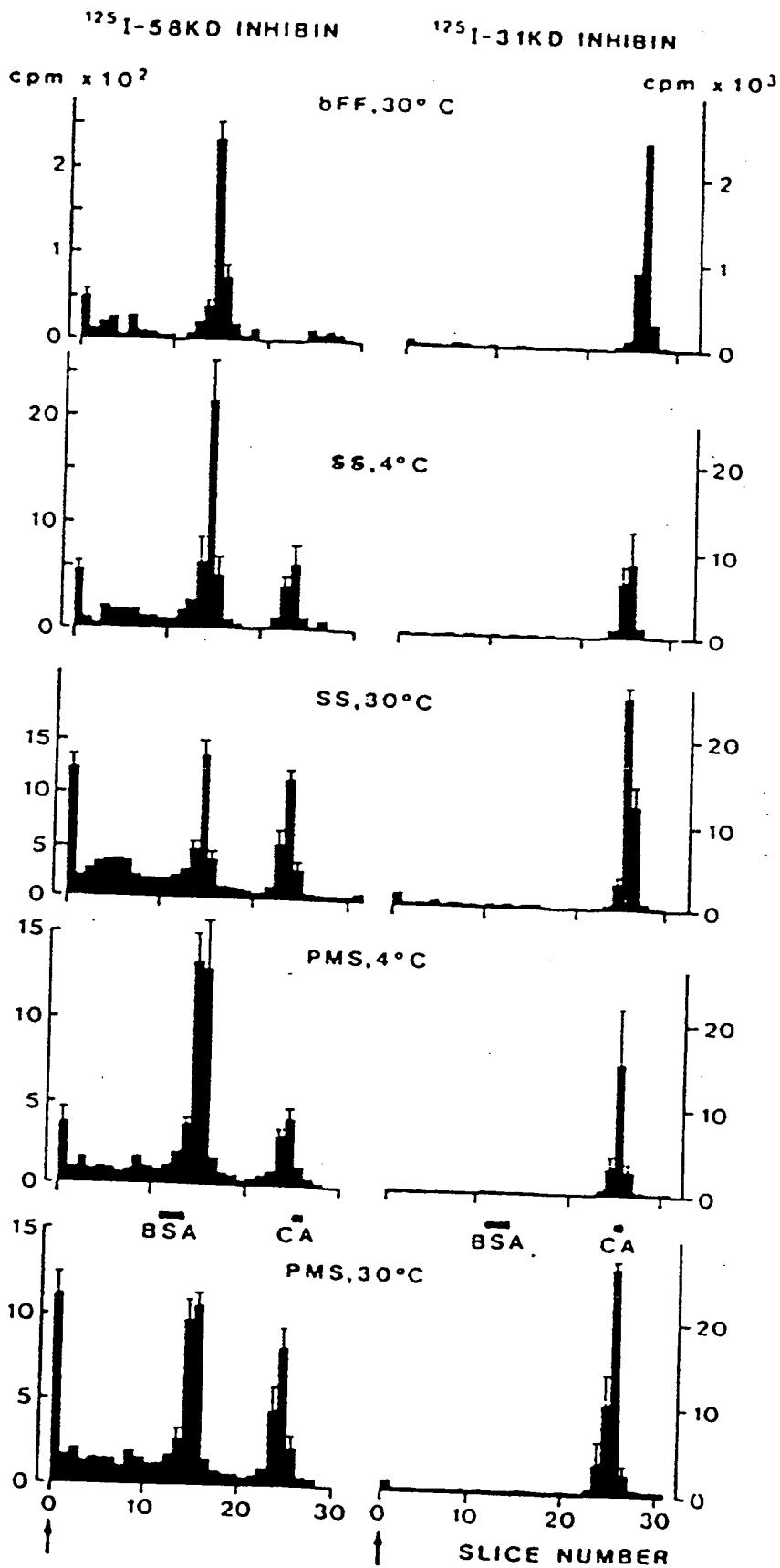
III. 4.

3/20

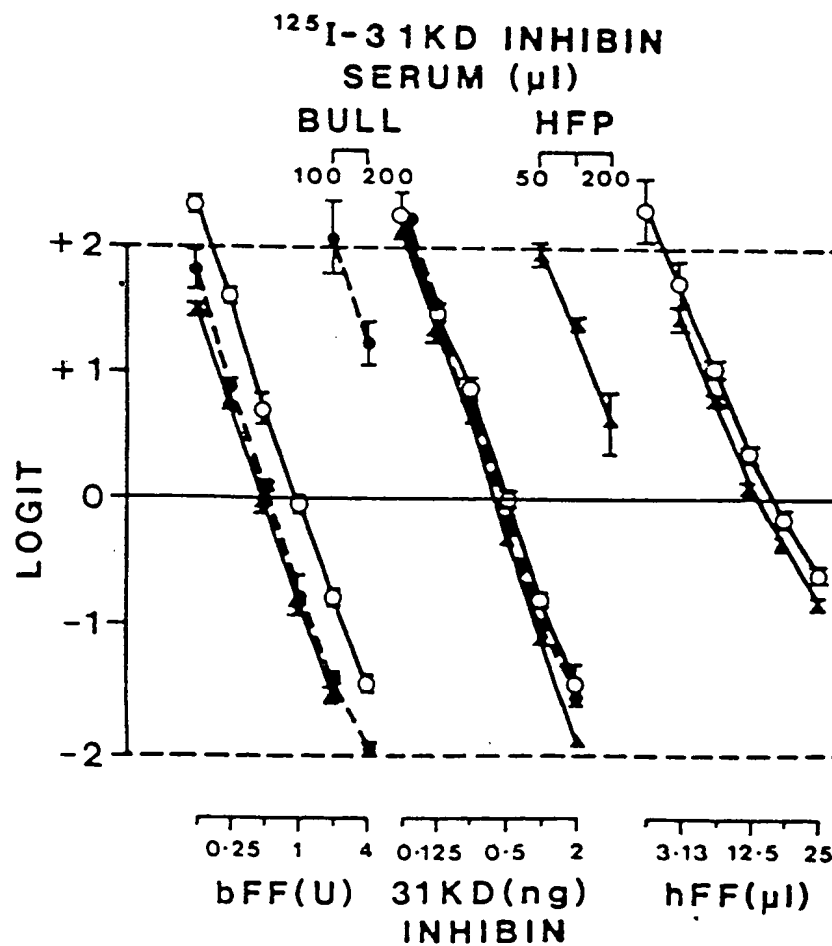
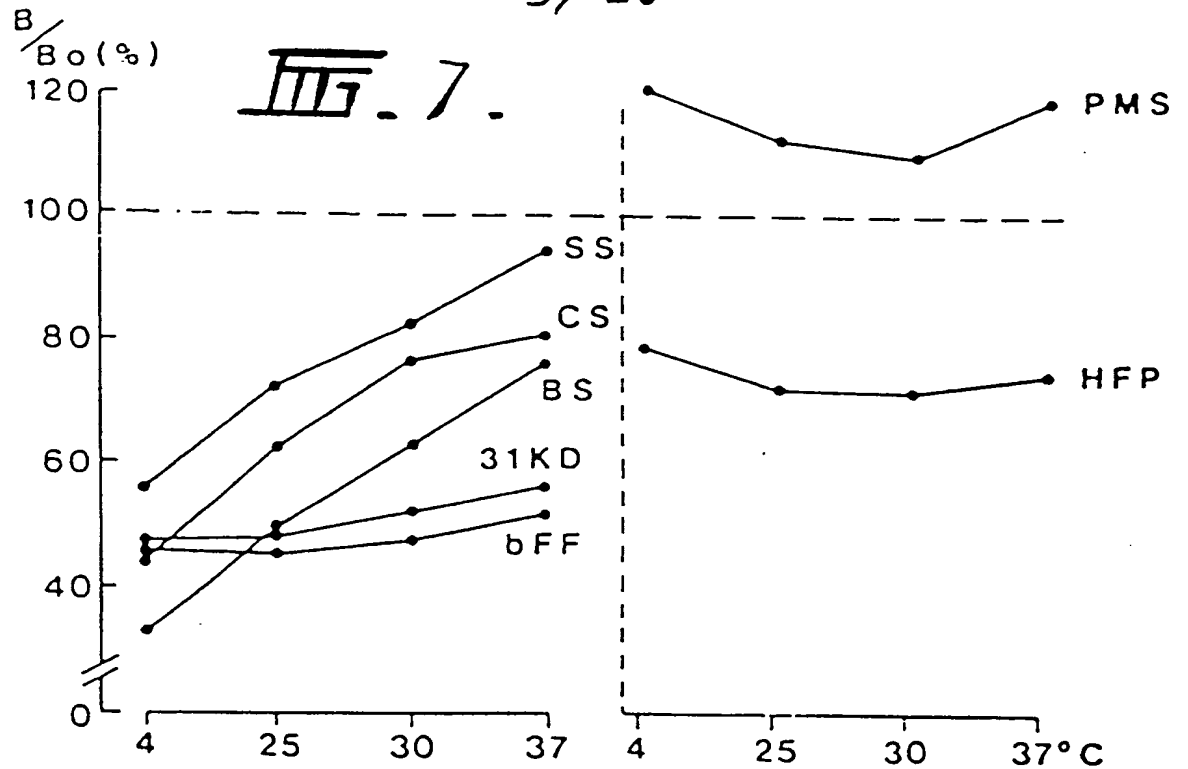
FIG. 5.

4/20

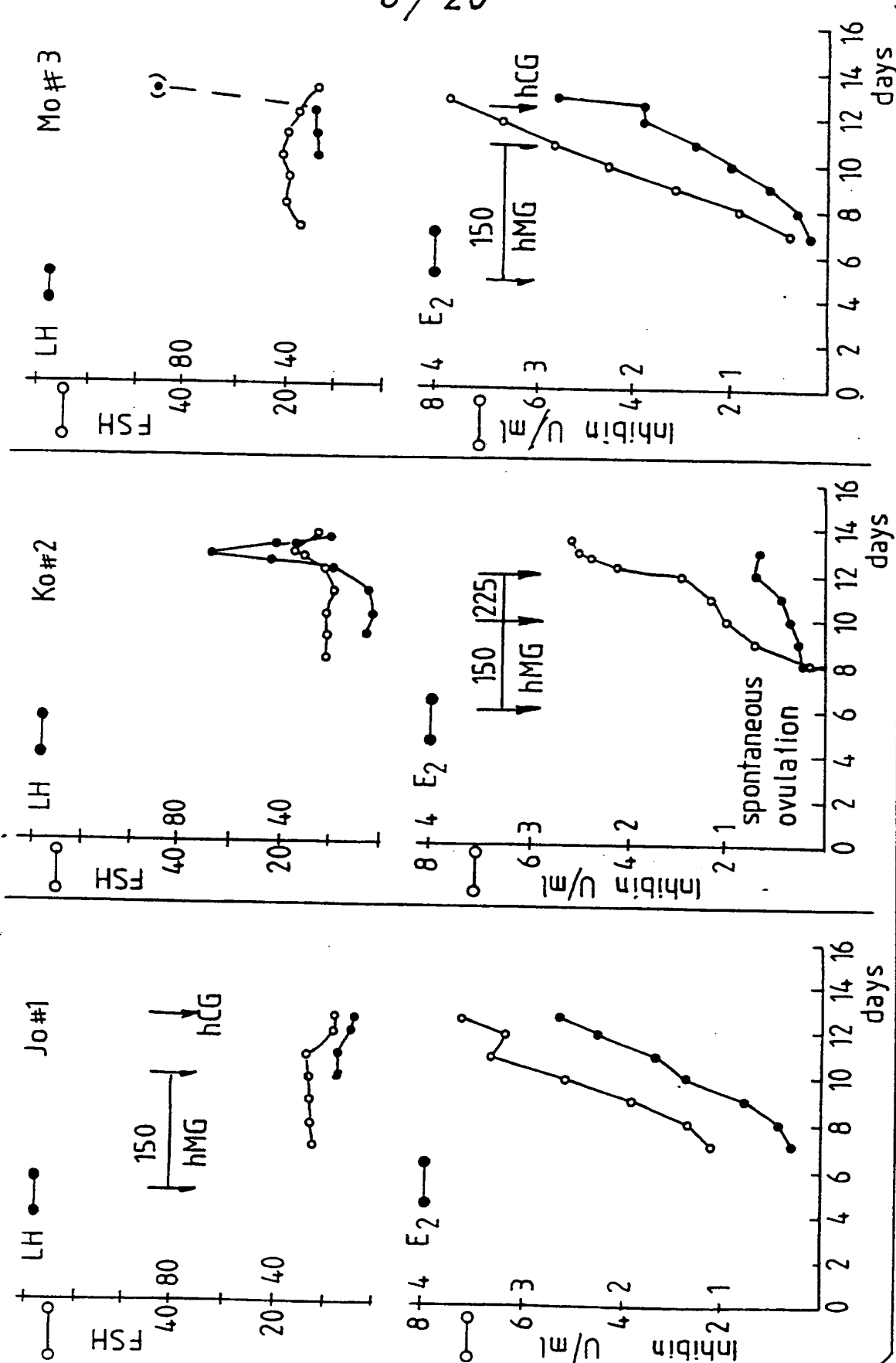
FIG. 6.



5/20



6/20

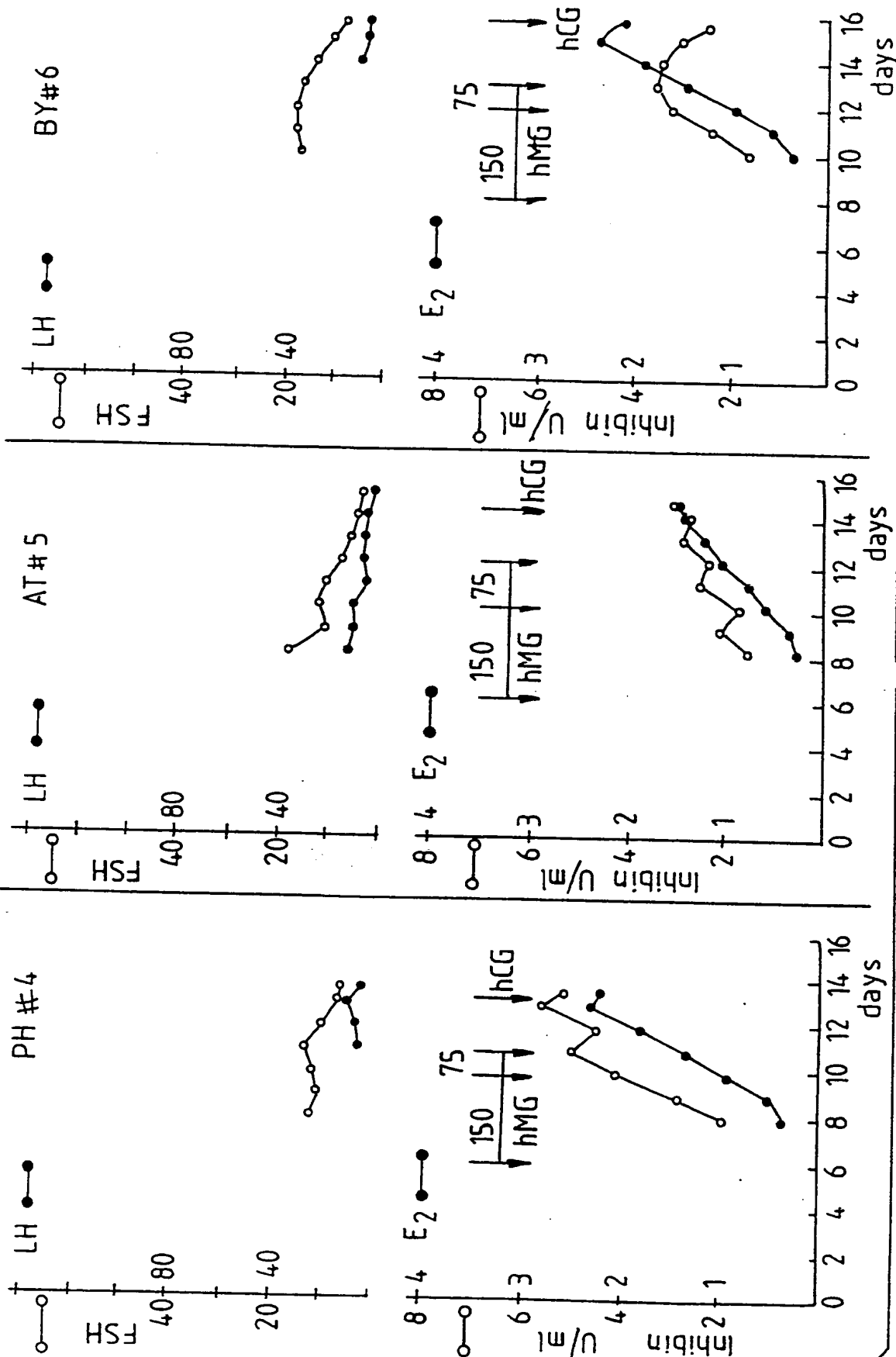


(a)

(b)

(c)

7/20

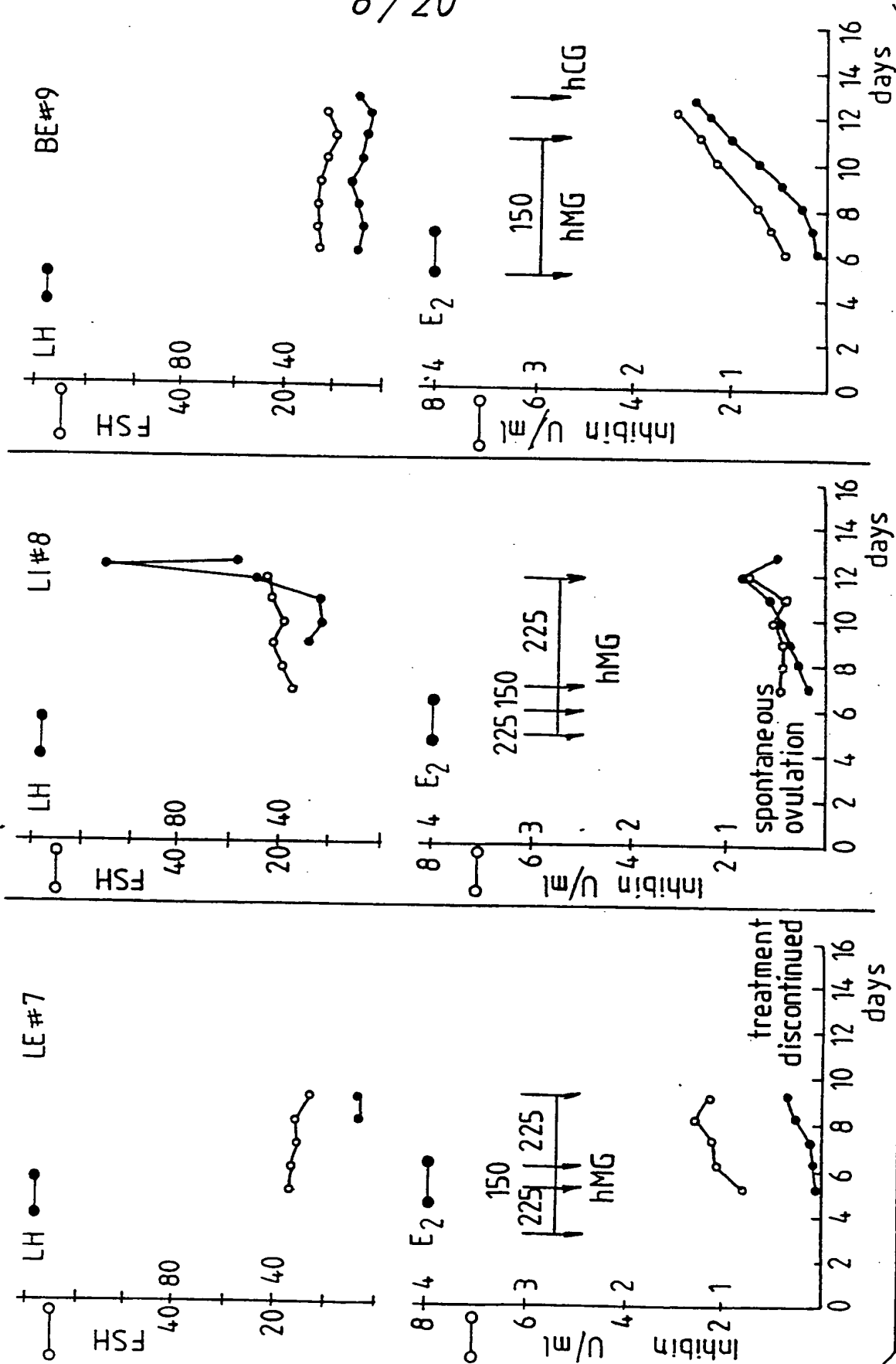


(d)

Fig. 9 (e)

(f)

8/20



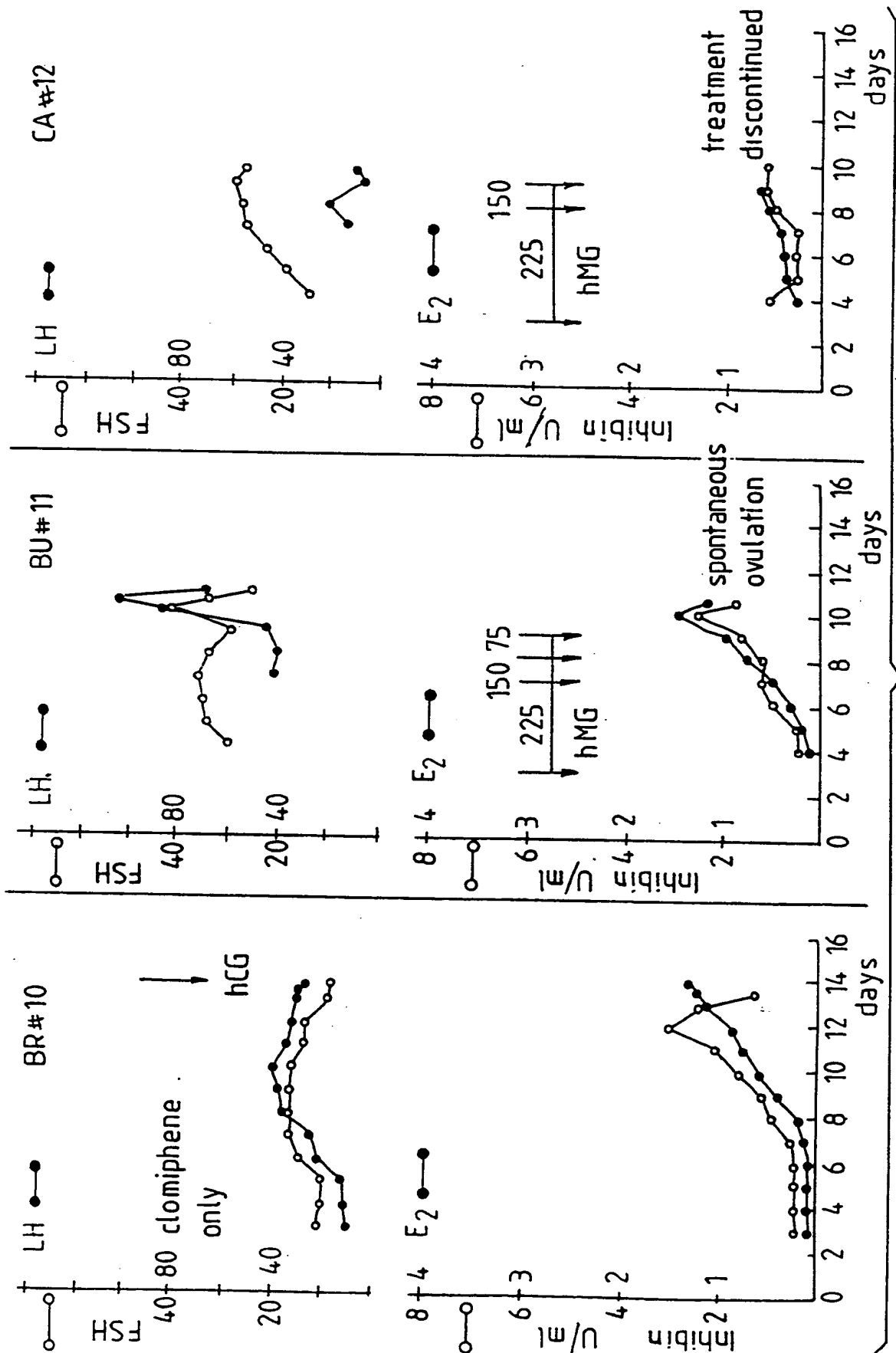
(i)

(h)

III. 9.

(g)

9/20

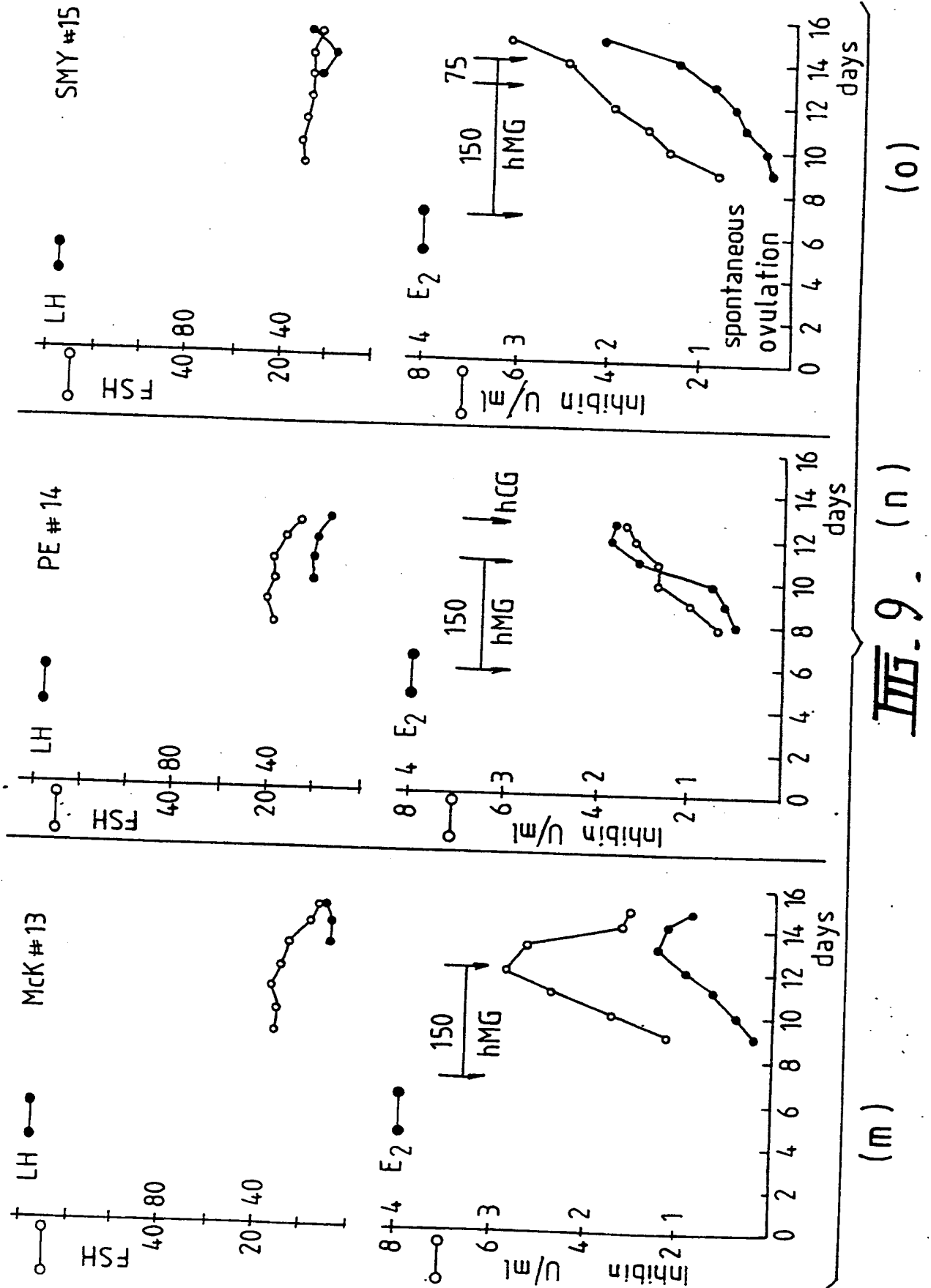


(j)

Fig. 9. (k)

(l)

10/20

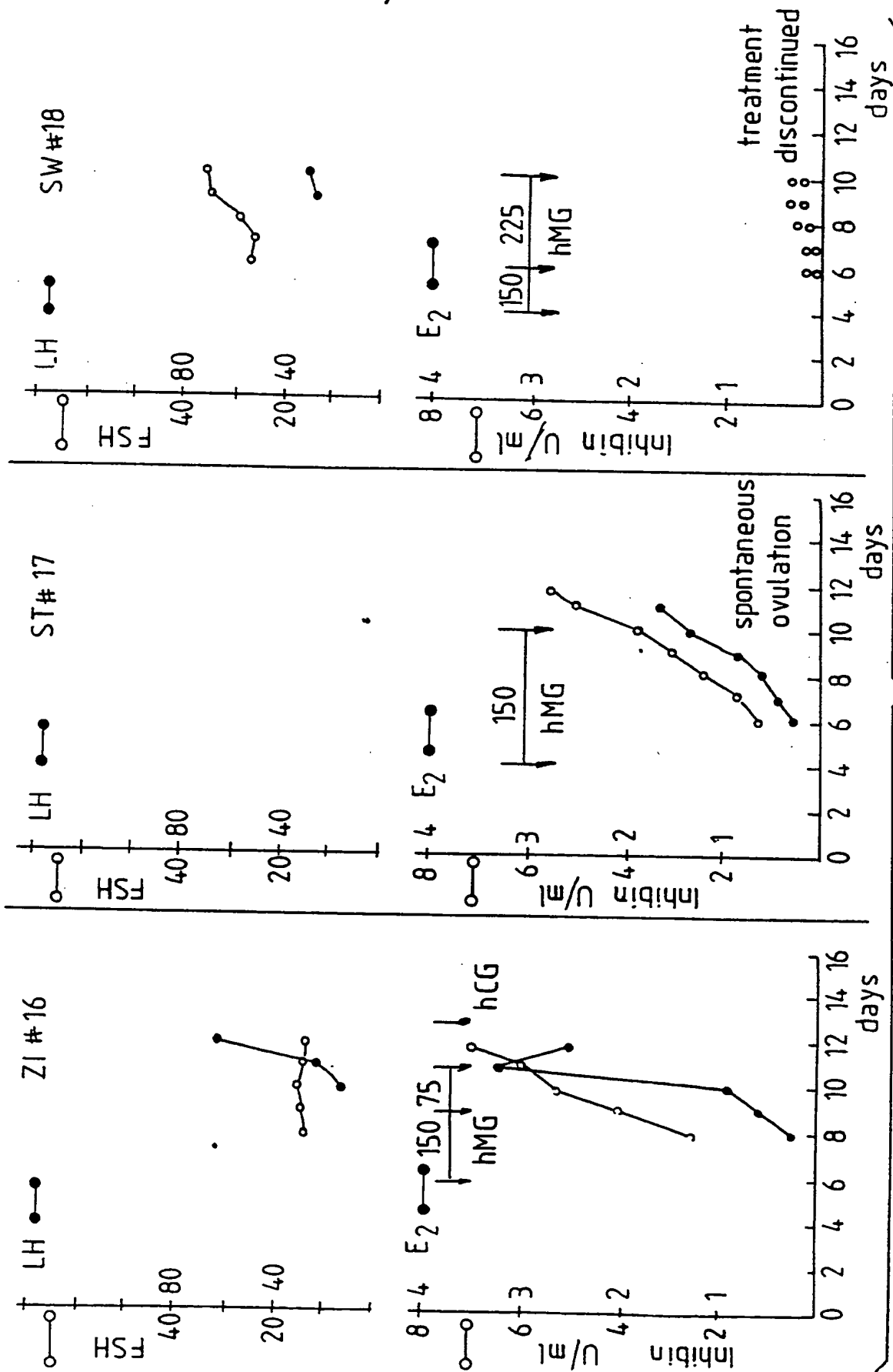


III. 9. (n)

(o)

(m)

11/20

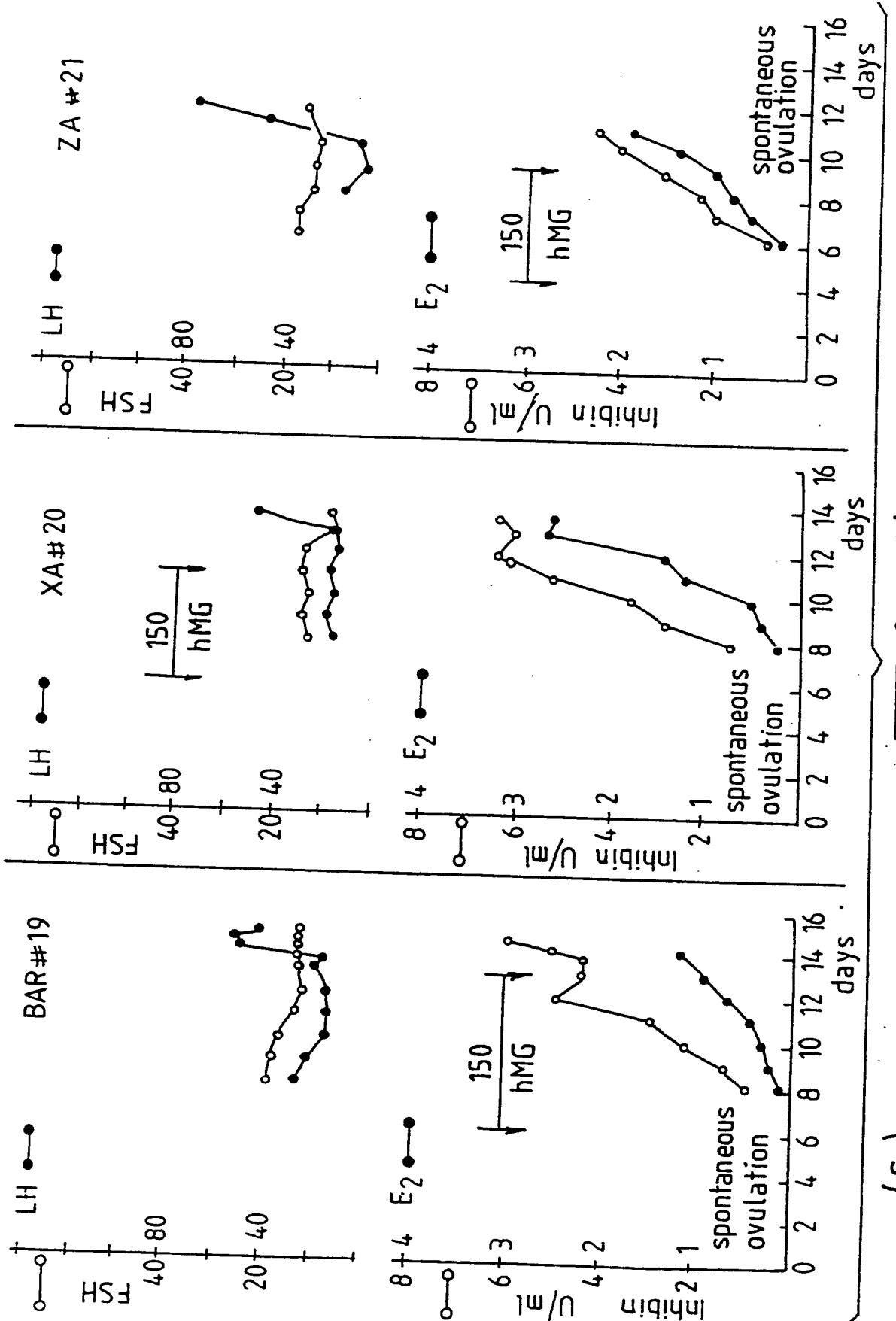


(r)

(q)

(p)

12 / 20

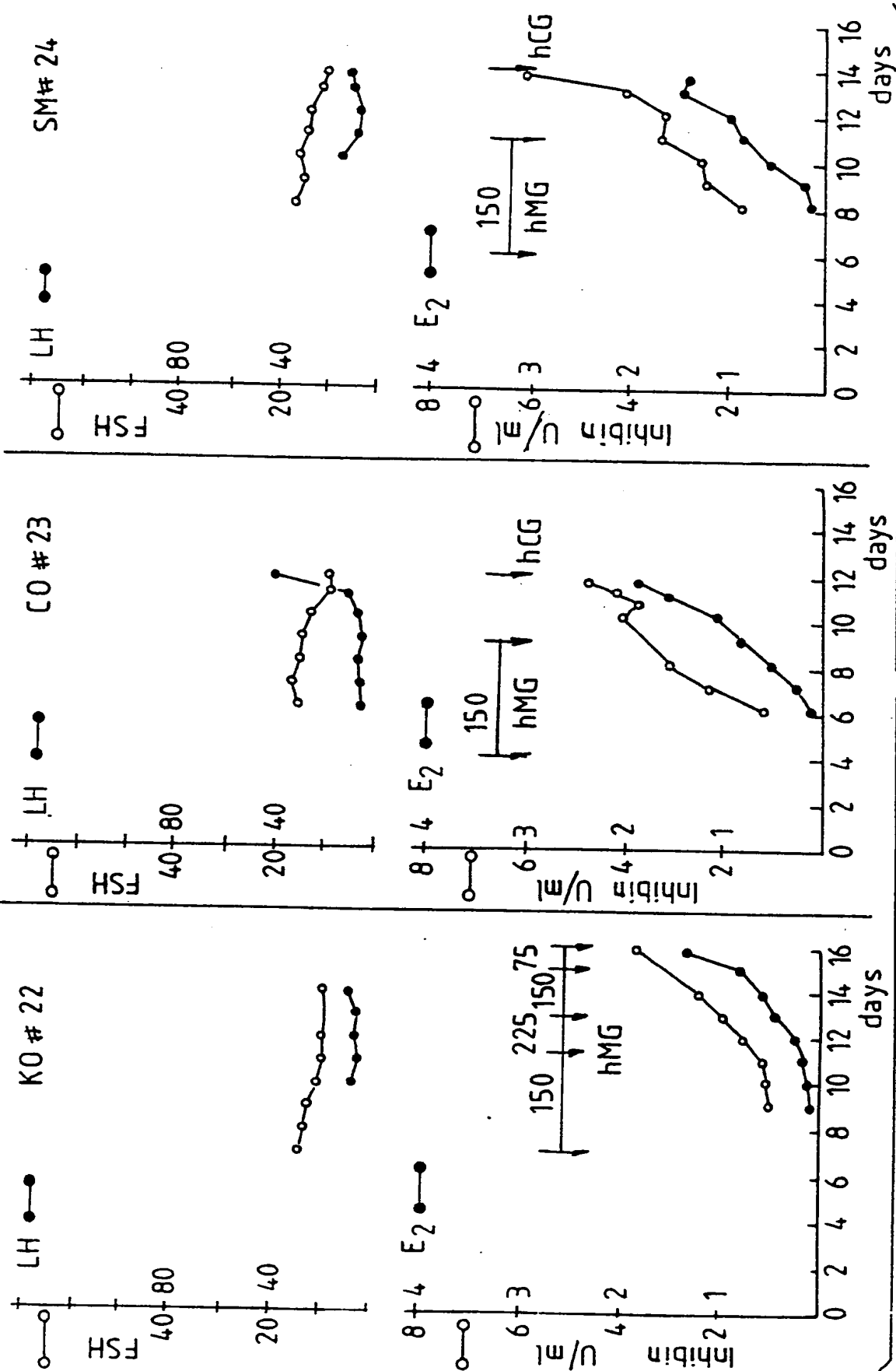


(s)

Fig. 9. (+)

(u)

13/20

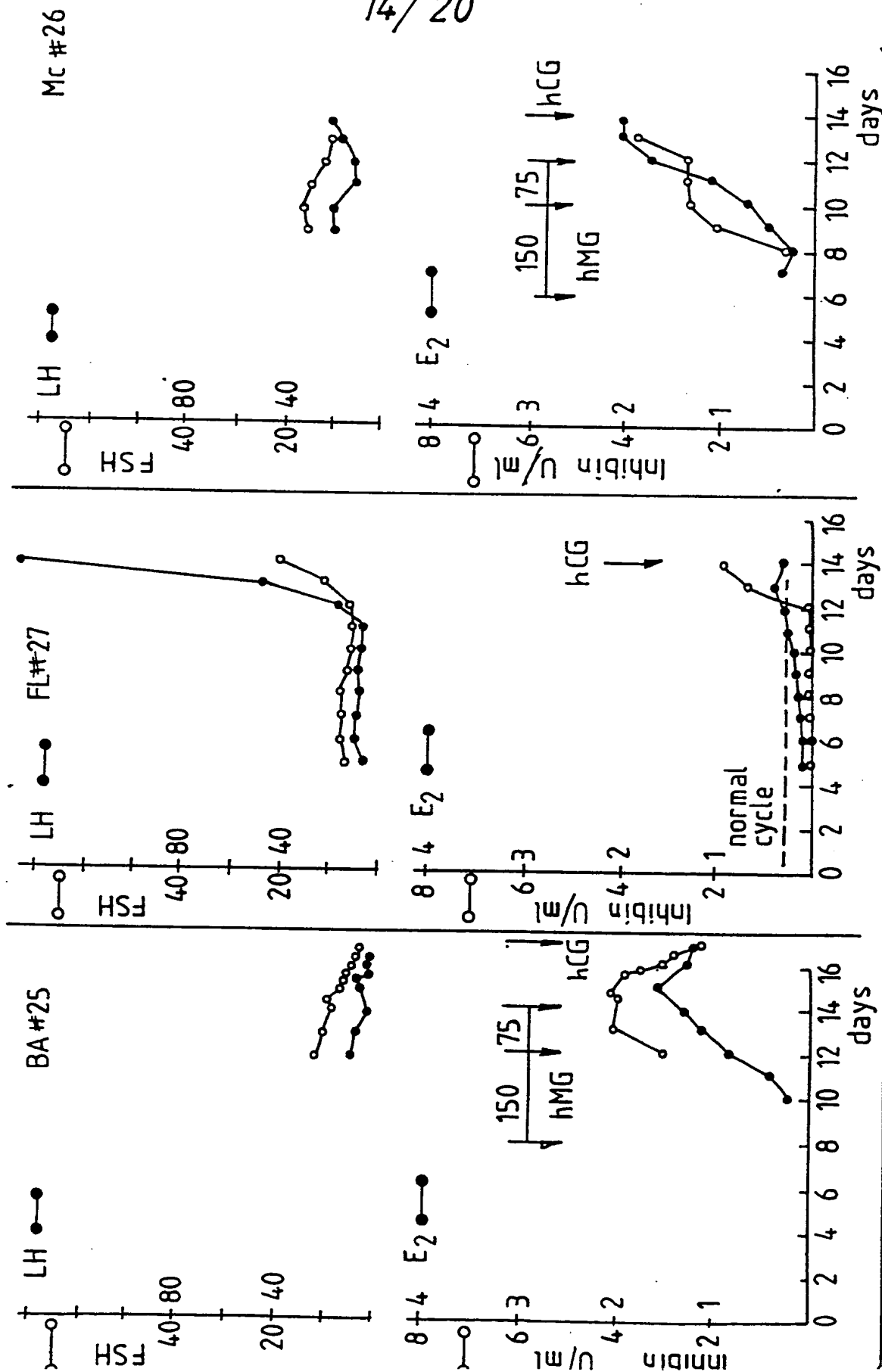


(x)

MG-9 (w)

(v)

14/20



(aa)

(z)

(y)

III.9.

15/20

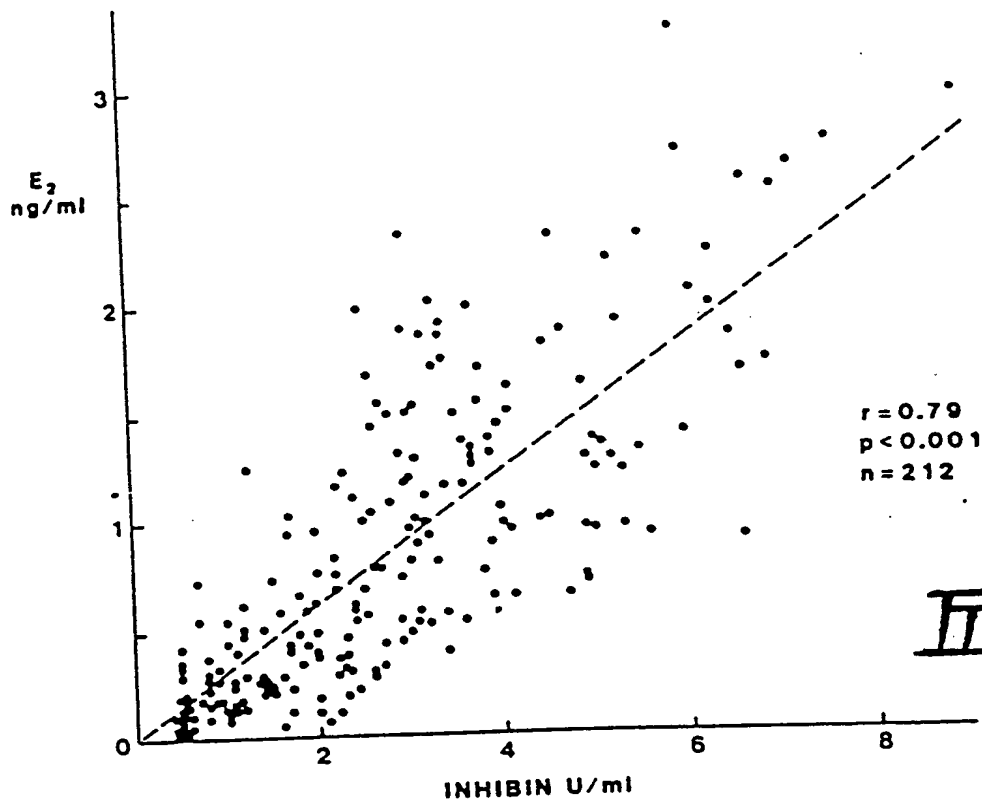


FIG. 10.

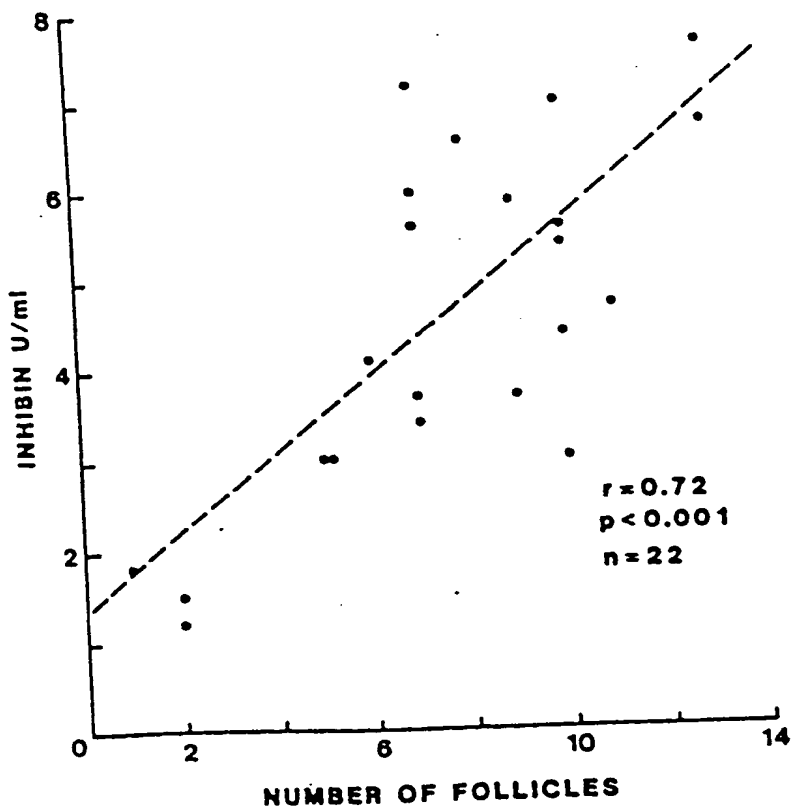
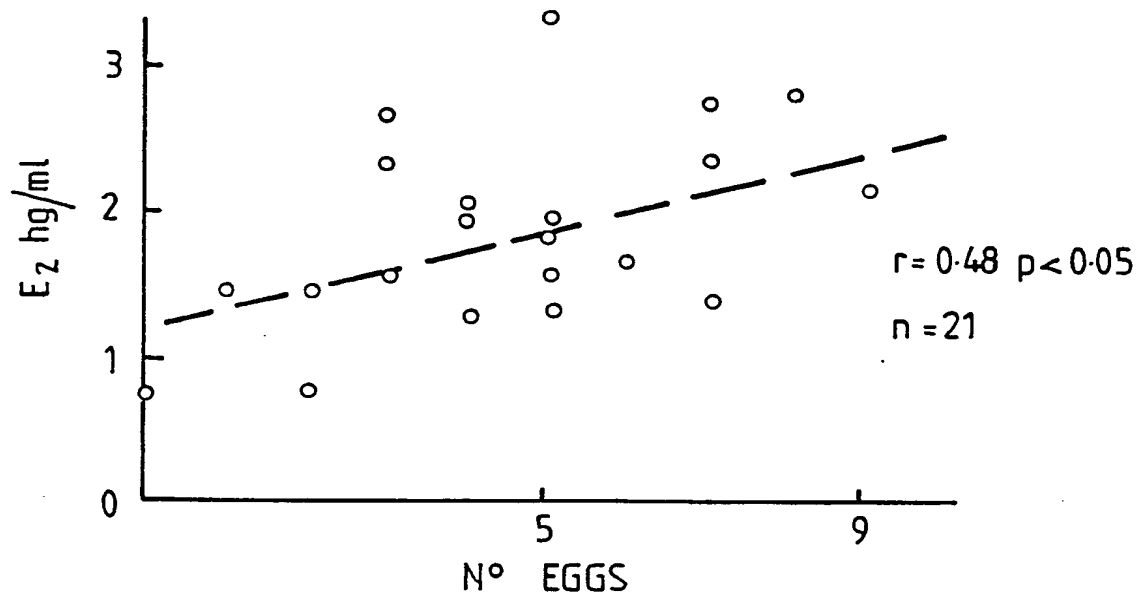


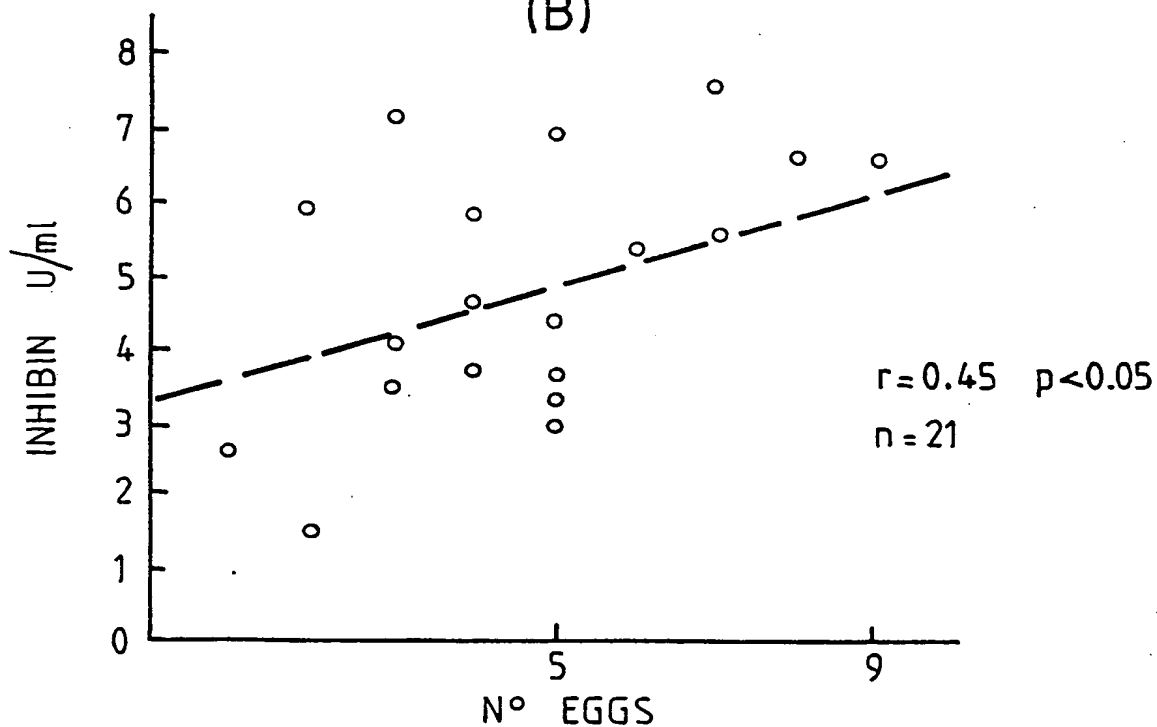
FIG. 12.

16/20

(A)

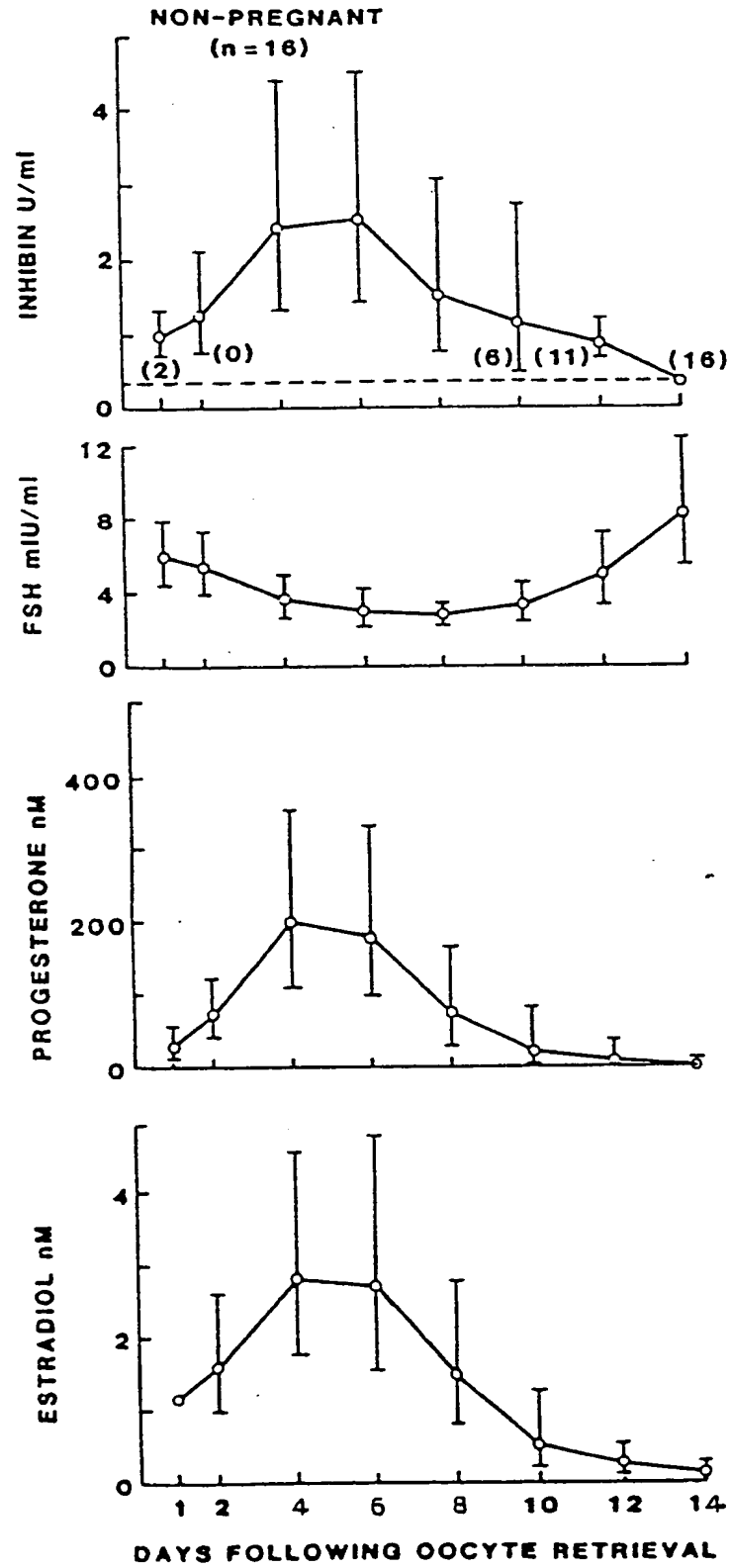


(B)

FIG. 11.

Comparison of eggs recovered at
Laparoscopy and Peak Plasma levels
of E_2 and INHIBIN

17/20

FIG. 13.

18 / 20

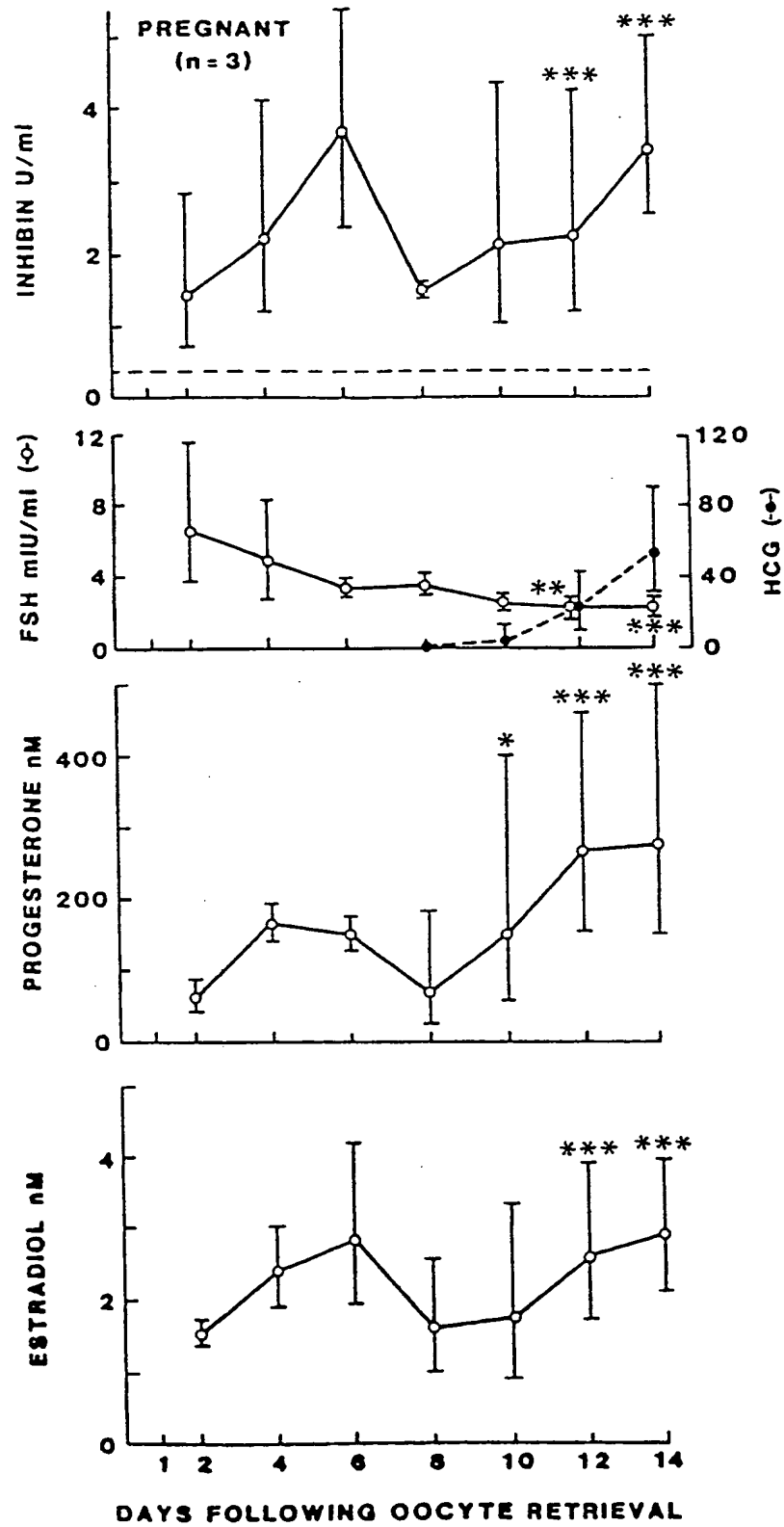
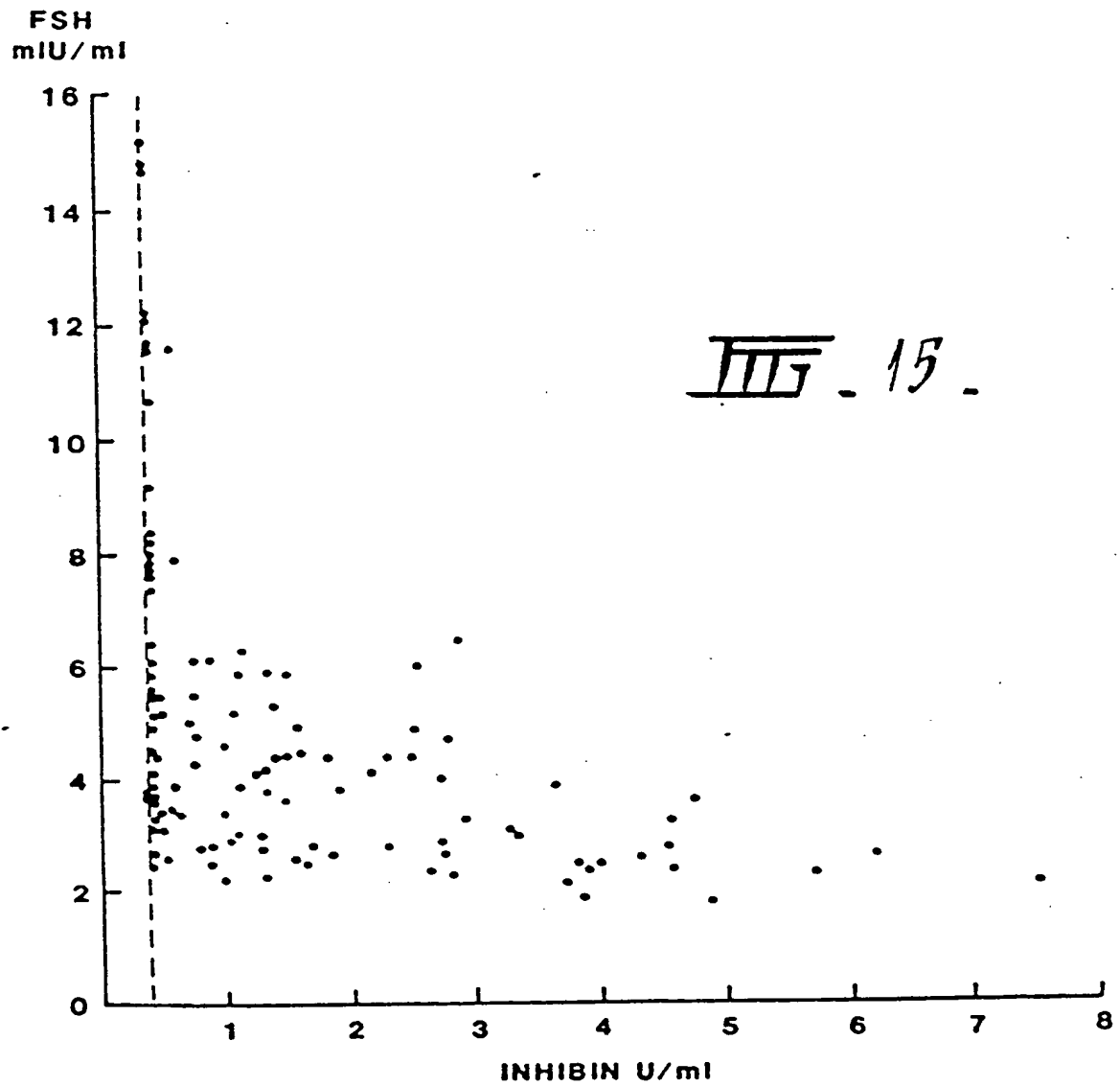
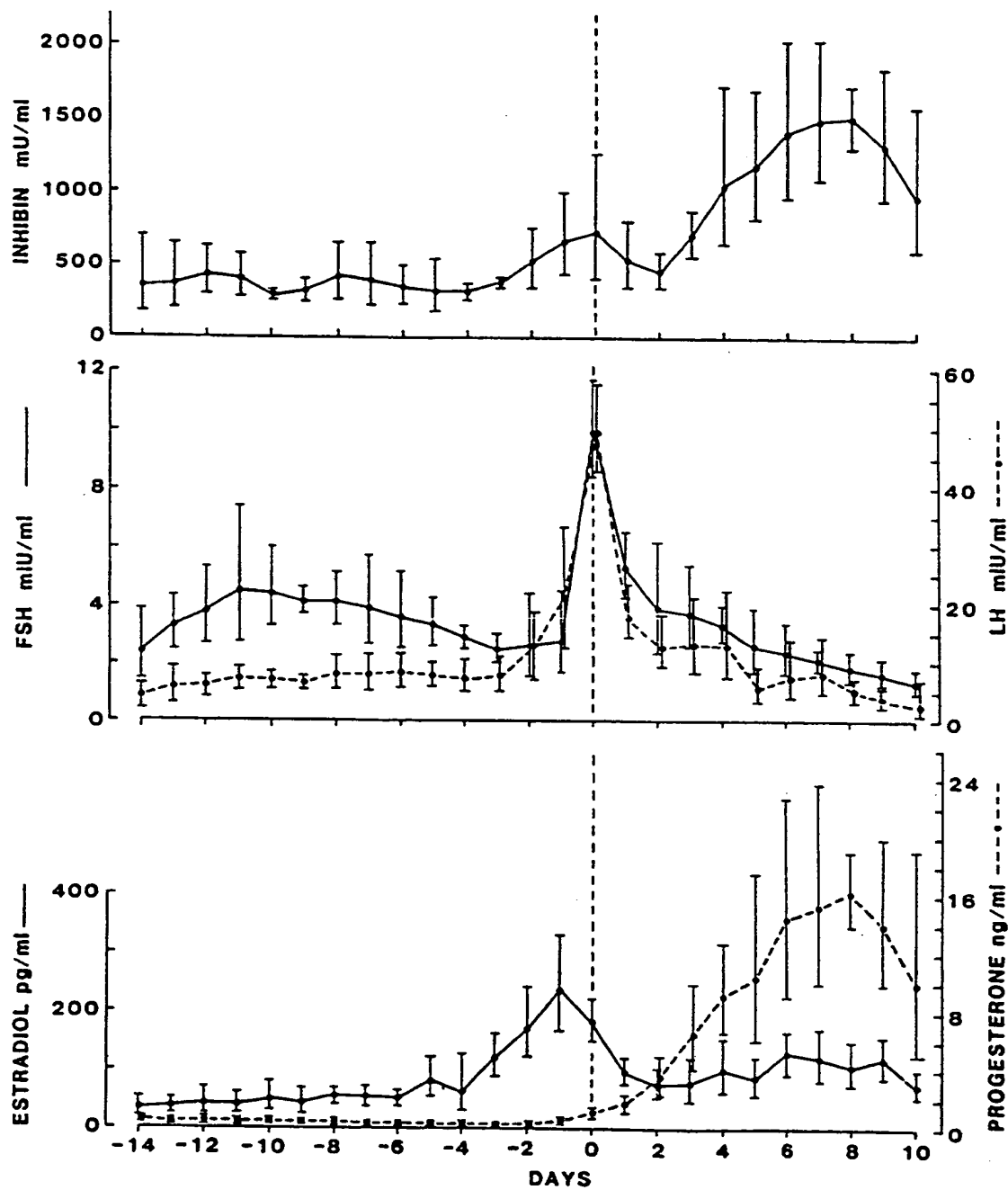


Fig. 14.

19/20



20/20

Fig. 16.

INTERNATIONAL SEARCH REPORT

International Application No PCT/AU 87/00070

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all)

According to International Patent Classification (IPC) or to both National Classification and IPC

Int. Cl.⁴ G01N 33/541, 33/74, C07K 15/12

II. FIELDS SEARCHED

Minimum Documentation Searched *

Classification System

Classification Symbols

IPC Derwent World Patent Index, Keyword "INHIBIN"

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched *

AU: IPC G01N 33/53, 33/577, 33/541, 33/54, 33/74, C07G 7/00, C07K 15/12

III. DOCUMENTS CONSIDERED TO BE RELEVANT *

Category *	Citation of Document, ** with indication, where appropriate, of the relevant passages **	Relevant to Claim No. **
X	International Journal of Gynaecology and Obstetrics, Volume 21, No.6, issued 1983 (Limerick, Ireland), S.P. Dandekar et al, "Levels of immunoreactive inhibin-like material in urine during the menstrual cycle", see pages 443-450.	ALL
X	S.M. McCann et al (Editors), Role of Peptides and Proteins in Control of Reproduction, Proceedings Workshop 1982, published 1983 by Elsevier (New York), F.H. DeJong et al, "Assay and Purification of Inhibin" see pages 257-273, especially pages 269-272.	ALL
X	Molecular and Cellular Endocrinology, Volume 44, No.3, issued 1986, March (Limerick, Ireland), D.M. Robertson et al, "Isolation of a 31 kDa form of inhibin from bovine follicular fluid", see pages 271-277, especially pages 273-276.	ALL
X,P	Molecular and Cellular Endocrinology, Volume 46, No.2, issued 1986, July (Limerick, Ireland), R.I. McLachlan et al, "The radioimmunoassay of bovine and human follicular fluid and serum inhibin", see pages 175-185.	ALL
X,P	WO,A, 86/06076 (BIOTECHNOLOGY AUSTRALIA PTY LTD et al) 23 October 1986 (23.10.86) see especially pages 47-53	ALL

continued

* Special categories of cited documents: **

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"Z" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search

22 June 1987 (22.06.87)

Date of Mailing of this International Search Report

(07.07.87) 7 JULY 1987

International Searching Authority

Australian Patent Office

Signature of Authorized Officer

J.G. Hanson

J.G. HANSON

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

X	WO,A, 86/00078 (MONASH UNIVERSITY et al) 3 January 1986 (03.01.86)	ALL
X	Biochemical and Biophysical Research Communications, Volume 133, No.1, issued 1985, November 27 (New York, New York), J. Rivier et al, "Purification and partial characterization of inhibin from porcine follicular fluid", see pages 120-127.	1,2,5
X,P	Biochemical and Biophysical Research Communications, Volume 136, No.3, issued 1985, May 14 (New York, New York), K. Miyamoto et al, "Demonstration of high molecular weight forms of inhibin in bovine follicular fluid (bFF) by using monoclonal antibodies to bFF 32K inhibin", see pages 1103-1109.	1,3,4,7

continued

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE ¹

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers..... because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claim numbers..... because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim numbers..... because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING ²

This international Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the international Searching Authority did not invite payment of any additional fee.

Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X,P	Journal of Endocrinology, Volume 109, No.3, issued 1986, June (Colchester, Essex, England), V. Lee et al, "Monoclonal antibody to rat ovarian inhibin", see pages 379-383.	1,4,5,7
X,P	Journal of Endocrinology, Volume 111, No.2, issued 1986, November (Colchester, Essex, England), S. Van Dijk et al, "Sexual dimorphism in immunoneutralization of rat and ovine inhibin", see pages 255-261.	1,2,3,5
X,E	AU,A, 63512/86 (GENENTECH INC.) 9 April 1987 (09.04.87) see page 50.	1,2,7
X	Advances in Experimental Medicine and Biology, Volume 147, issued 1982 (New York, New York), F.H. De Jong et al, "Purification, characterization, and in vitro production of inhibin", see pages 37-52 especially pages 42-46.	1,2,3,5,6,7
X	Biological Research in Pregnancy and Perinatology, Volume 4, No.3, issued 1983 (Munich-Deisenhofen, West Germany), J.J. Sheth et al, "Bioimmunoreactive inhibin-like substance in human fetal gonads", see pages 110-112.	1,2,3,7,8,9
X	Journal of Biosciences, Volume 7, No.2, issued 1985, March (India), A.H. Bandivdekar et al, "Isolation of inhibin-like peptides from human placenta", see pages 175-190.	1,2,3,5
X	Chemical Abstracts, Volume 102, No.17, issued 1985, April 29 (Columbus, Ohio, USA) X. Lu et al, "Preparation of inhibin from porcine semen and its radioimmunoassay", see page 64, column 1, abstract No.143 300h, Shengzhi Yu Biyun, 1984 4(4), 27-33 (Chin)	1,2,7
X	Indian Journal of Experimental Biology, Volume 23, No.10, issued 1985, October (New Delhi, India), K.S. Hurkadli et al, "Studies on immunoneutralization of inhibin: a time course study", see pages 561-565.	1,2,3,5,7
X,P	US,A, 4624944 (LI et al) 25 November 1986 (25.11.86) see columns 7-9.	1,2,3,7

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON
INTERNATIONAL APPLICATION NO. PCT/AU 87/00070

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document
Cited in Search
Report

Patent Family Members

AU 63512/86

EP 222491

IL 80218

WO 8606076

AU 59039/86
IL 78519

DK 6113/86

EP 218717

WO 8600078

AU 44374/85
IL 75412

DK 605/86
NO 860427

EP 185034
ZA 8504346

END OF ANNEX

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
25 April 2002 (25.04.2002)

PCT

(10) International Publication Number
WO 02/33090 A2

- (51) International Patent Classification⁷: **C12N 15/16**,
C07K 14/575, 14/435, A61K 38/22, 31/7088
- (21) International Application Number: PCT/CA01/01463
- (22) International Filing Date: 15 October 2001 (15.10.2001)
- (25) Filing Language: English
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- (30) Priority Data:
2,321,256 16 October 2000 (16.10.2000) CA
2,355,334 20 August 2001 (20.08.2001) CA
- (71) Applicant: **PROCYON BIOPHARMA INC.** [CA/CA];
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2500, 1100, René-Lévesque Boulevard West, Montreal,
Quebec H3B 5C9 (CA).
- (81) Designated States (*national*): AE, AG, AL, AM, AT, AU,
AZ, BA, BB, BG, BR, BY, BZ, CH, CN, CO, CR, CU, CZ,
DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM,
HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK,
LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX,
MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL,
TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW.
- (84) Designated States (*regional*): ARIPO patent (GH, GM,
KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian
patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European
patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE,
IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF,
CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD,
TG).
- Published:**
— *without international search report and to be republished
upon receipt of that report*
- For two-letter codes and other abbreviations, refer to the "Guid-
ance Notes on Codes and Abbreviations" appearing at the begin-
ning of each regular issue of the PCT Gazette.*

(54) Title: PHARMACEUTICAL PREPARATIONS AND METHODS FOR INHIBITING TUMORS

(57) Abstract: The invention provides pharmaceutical compositions and method for inhibiting growth of prostatic adenocarcinoma, stomach cancer, breast cancer, endometrial, ovarian or other cancers of epithelial secretion, or benign prostate hyperplasia (BPH). In one embodiment the pharmaceutical composition includes human rHuPSP94, antigenic portions thereof, and functionally equivalent polypeptides thereof. In another embodiment, the pharmaceutical composition includes a mixture of human rHuPSP94, antigenic portions thereof, and functionally equivalent polypeptides thereof and an anticancer drug which may be administered in an appropriate dosage form, dosage quantity and dosage regimen to a patient suffering from, for example of prostatic adenocarcinoma, stomach cancer, breast cancer, endometrial, ovarian or other cancers of epithelial secretion, benign prostate hyperplasia, or (BPH) gastrointestinal cancer. The anticancer drug of the latter mixture may be one selected from the group of drugs including mitomycin, idarubicin, cisplatin, 5-fluoro-uracil, methotrexate, adriamycin, daunomycin, taxol, taxol derivative, and mixtures thereof.

WO 02/33090 A2

PHARMACEUTICAL PREPARATIONS AND METHODS
FOR INHIBITING TUMORS

5

FIELD OF THE INVENTION

The present invention relates to pharmaceutical preparations (i.e., composition) for use as tumor suppressive agents for tumors arising from cancers such as prostatic adenocarcinoma, stomach
10 cancer, breast cancer, endometrial and ovarian cancers, and benign prostate hyperplasia (BPH).

BACKGROUND OF THE INVENTION

15 The prostate gland, which is found exclusively in male mammals, produces several components of semen and blood and several regulatory peptides. The prostate gland comprises stroma and epithelium cells, the latter group consisting of columnar secretory cells and basal nonsecretory cells. A proliferation of these basal cells as well as
20 stroma cells gives rise to benign prostatic hyperplasia (BPH), which is one common prostate disease. Another common prostate disease is prostatic adenocarcinoma (CaP), which is the most common of the fatal pathophysiological prostate cancers, and involves a malignant transformation of epithelial cells in the peripheral region of the
25 prostate gland. Prostatic adenocarcinoma and benign prostatic hyperplasia are two common prostate diseases, which have a high rate of incidence in the aging human male population. Approximately one out of every four males above the age of 55 suffers from a prostate disease of some form or another. Prostate cancer is the second most
30 common cause of cancer related death in elderly men, with approximately 96,000 cases diagnosed and about 26,000 deaths reported annually in the United States.

35 Studies of the various substances synthesized and secreted by normal, benign and cancerous prostates carried out in order to gain an understanding of the pathogenesis of the various prostate diseases reveal that certain of these substances may be used as immunohistochemical tumor markers in the diagnosis of prostate disease. The three predominant proteins or polypeptides secreted by
40 a normal prostate gland are: (1) Prostatic Acid Phosphatase (PAP); (2) Prostate Specific Antigen (PSA); and, (3) Prostate Secretory Protein of 94 amino acids (PSP94), which is also known as Prostatic Inhibin Peptide (PIP), Human Seminal Plasma Inhibin (HSPI), or β -

microseminoprotein (β -MSP), and which is hereinafter referred to as PSP94.

PSP94 is a simple non-glycosylated cysteine-rich protein, and constitutes one of three predominant proteins found in human seminal fluid along with Prostate Specific Antigen (PSA) and Prostate Acid Phosphatase (PAP). PSP94 has a molecular weight of 10.7 kiloDalton (kDa), and the complete amino acid sequence of this protein has already been determined (SEQ ID NO:1). The cDNA and gene for PSP94 have been cloned and characterized (Ulvback, et al., Biochem. Biophys. Res. Comm., 164:1310, 1989; Green, et al., Biochem. Biophys. Res. Comm., 167:1184, 1990). Immunochemical and in situ hybridization techniques have shown that PSP94 is located predominantly in prostate epithelial cells. It is also present, however, in a variety of other secretory epithelial cells (Weiber, et al., Am. J. Pathol., 137:593, 1990). PSP94 has been shown to be expressed in prostate adenocarcinoma cell line, LNCap (Yang, et al., J. Urol., 160:2240, 1998). As well, an inhibitory effect of exogenous PSP94 on tumor cell growth has been observed both in vivo and in vitro (Garde, et al., Prostate, 22:225, 1993; Lokeshwar, et al., Cancer Res., 53:4855, 1993), suggesting that PSP94 could be a negative regulator for prostate carcinoma growth via interaction with cognate receptors on tumor cells.

Native PSP94 has been shown to have a therapeutic modality in treating hormone refractory prostate cancer (and potentially other prostate indications).

Metabolic and immunohistochemical studies have shown that the prostate is a major source of PSP94. PSP94 is involved in the feedback control of, and acts to suppress secretion of, circulating follicle-stimulating hormone (FSH) both in-vitro and in-vivo in adult male rats. PSP94 acts both at the pituitary as well as at the prostate site since both are provided with receptor sites for PSP94. It has been demonstrated to suppress the biosynthesis and release of FSH from the rat pituitary as well as to possibly affect the synthesis/secretion of an FSH-like peptide by the prostate. These findings suggest that the effects of PSP-94 on tumor growth in vivo, could be attributed to the reduction in serum FSH levels.

Both PSA and PAP have been studied as tumor markers in the detection of prostate disease, but since both exhibit elevated levels in prostates having benign prostatic hyperplasia (BPH), neither marker is specific and therefore they are of limited utility.

Recently, it has been shown that PSP94 concentrations in serum of patients with BPH or CaP are significantly higher than normal. The highest serum concentration of PSP94 observed in normal men is approximately 40 ng/ml, while in men with either BPH or CaP, serum concentrations of PSP94 have been observed in the range from 300-400 ng/ml. Because there exists some overlap in the concentrations of PSP94 in subjects having normal prostates and patients exhibiting either BPH or CaP, serum levels in and of themselves are of little value.

A major therapy in the treatment of prostate cancer is androgen-ablation. While most patients respond initially to this treatment, its effectiveness decreases over time, possibly because of the presence of a heterogenous population of androgen-dependant and androgen-independent cells to the androgen treatment, while any androgen insensitive cells present would continue to proliferate unabated.

Other forms of cancer, which are currently exacting a heavy toll on population are breast cancer in women and cancer of the gastrointestinal tract. Currently, the use of various cancer drugs such as mitomycin, idarubicin, cisplatin, 5-fluoro-uracil, methotrexate, adriamycin and daunomycin form part of the therapy for treating such cancers. One drawback to such a therapeutic treatment is the presence of adverse side effects due to the drugs in the concentration ranges required for effective treatment.

Accordingly, it would be advantageous to find a more effective means of arresting the growth of prostate, breast and gastrointestinal cancer cells and tumors, which may be used effectively against both androgen sensitive and androgen insensitive cells.

In previous work, described in United States Patent No. 5,428,011, we provided pharmaceutical preparations (i.e., compositions) of native human seminal plasma PSP94 for inhibiting in-vitro and in-vivo cancerous prostate, gastrointestinal and breast tumors. The pharmaceutical preparations included native human seminal plasma PSP94 which could be administered in an appropriate dosage form, dosage quantity and dosage regimen to a patient suffering from prostate cancer. In another embodiment, the pharmaceutical preparation included a mixture of human seminal plasma PSP94 and an anticancer drug which may be administered in an

appropriate dosage form, dosage quantity and dosage regimen to a patient suffering from, for example gastrointestinal cancer.

5 PSP94 sourced from human seminal fluid carries with it significant risk of contamination with infectious agents (e.g., HIV, hepatitis (a, b, or c), and other viruses and/or prions). Even with the use of harsh chemical treatment, total eradication of such agents cannot be guaranteed. Additionally, human seminal fluid is found in limited supply, thus making bulk production of PSP94 very difficult.
10 Therefore, the acceptability of human or even xenogeneic sourced PSP94 may be very difficult for both the regulatory authorities and the marketplace.

Therefore, the use of recombinant technology for producing
15 PSP94 would represent a significant advancement, as recombinant PSP94 could be produced both free of pathogens and in an unlimited supply. Furthermore, the material would be homogeneous from a single lot source, avoiding batch variation.

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SUMMARY OF THE INVENTION

In its first aspect the present invention relates to a polypeptide or a polypeptide analog selected from the group
25 consisting of the polypeptide as set forth in SEQ ID NO: 3, the polypeptide as set forth in SEQ ID NO: 4, the polypeptide as set forth in SEQ ID NO: 5, and the polypeptide as set forth in SEQ ID NO: 6, a polypeptide analog of at least five contiguous amino acids of SEQ ID NO: 2, of SEQ ID NO: 3, of SEQ ID NO: 4, of SEQ ID NO: 5, or
30 of SEQ ID NO: 6, a polypeptide analog of at least two contiguous amino acids of SEQ ID NO: 2, of SEQ ID NO: 3, of SEQ ID NO: 4, of SEQ ID NO: 5, or of SEQ ID NO: 6, a polypeptide analog consisting of the amino acid sequence $X_1 W Q X_2 D X_1 C X_1 X_2 C X_2 C X_3 X_1 X_2$ as set forth in SEQ ID NO: 89, wherein X_1 is either glutamic acid (Glu), asparagine
35 (Asn) or aspartic acid (Asp), X_2 is either threonine (Thr) or serine (Ser), and X_3 is either tyrosine (Tyr) or phenylalanine (Phe), a polypeptide analog comprising SEQ ID NO: 5 and having an addition of at least one amino acid to its amino-terminus, wherein said polypeptide analog comprising SEQ ID NO: 5 is selected from the group
40 consisting of SEQ ID NO: 59 to SEQ ID NO: 88, a polypeptide analog comprising SEQ ID NO: 5 and having an addition of at least one amino acid to its carboxy-terminus, wherein said polypeptide analog comprising SEQ ID NO: 5 is selected from the group consisting of SEQ ID NO: 10 to SEQ ID NO: 58, a polypeptide analog comprising two to

fifty units of SEQ ID NO: 5, a polypeptide analog comprising two to ten units of SEQ ID NO: 5, a polypeptide analog consisting of a sequence of from two to fourteen amino acid units wherein the amino acid units are selected from the group of amino acid units of SEQ ID NO: 5 consisting of glutamic acid (Glu), tryptophan (Trp), glutamine (Gln), threonine (Thr), aspartic acid (Asp), asparagine (Asn), cysteine (Cys), or tyrosine (Tyr), a polypeptide analog having at least 90 % of its amino acid sequence identical to the amino acid sequence set forth in SEQ ID NO: 5, a polypeptide analog having at least 70 % of its amino acid sequence identical to the amino acid sequence set forth in SEQ ID NO: 5, and a polypeptide analog having at least 50 % of its amino acid sequence identical to the amino acid sequence set forth in SEQ ID NO: 5. The polypeptide analog mentioned herein may be capable of inhibiting the growth of a tumor or more precisely may be capable of inhibiting the growth of prostatic adenocarcinoma, stomach cancer, breast cancer, endometrial, ovarian or other cancers of epithelial secretion, or benign prostate hyperplasia (BPH).

In a second aspect, the present invention relates to the use of a polypeptide or a polypeptide analog selected from the group consisting of rHuPSP94 as set forth in SEQ ID NO: 2, the decapeptide as set forth in SEQ ID NO: 3, the polypeptide as set forth in SEQ ID NO: 4 (polypeptide 7-21), the polypeptide as set forth in SEQ ID NO: 5 (PCK3145), and the polypeptide as set forth in SEQ ID NO: 6 (polypeptide 76-94), a polypeptide analog of at least five contiguous amino acids of SEQ ID NO: 2, of SEQ ID NO: 3, of SEQ ID NO: 4, of SEQ ID NO: 5, or of SEQ ID NO: 6, a polypeptide analog of at least two contiguous amino acids of SEQ ID NO: 2, of SEQ ID NO: 3, of SEQ ID NO: 4, of SEQ ID NO: 5, or of SEQ ID NO: 6, a polypeptide analog consisting of the amino acid sequence $X_1 W Q X_2 D X_1 C X_1 X_2 C X_2 C X_3 X_1 X_2$ as set forth in SEQ ID NO: 89, wherein X_1 is either glutamic acid (Glu), asparagine (Asn) or aspartic acid (Asp), X_2 is either threonine (Thr) or serine (Ser), and X_3 is either tyrosine (Tyr) or phenylalanine (Phe), a polypeptide analog comprising SEQ ID NO: 5 and having an addition of at least one amino acid to its amino-terminus, wherein said polypeptide analog comprising SEQ ID NO: 5 is selected from the group consisting of SEQ ID NO: 59 to SEQ ID NO: 88, a polypeptide analog comprising SEQ ID NO: 5 and having an addition of at least one amino acid to its carboxy-terminus, wherein said polypeptide analog comprising SEQ ID NO: 5 is selected from the group consisting of SEQ ID NO: 10 to SEQ ID NO: 58, a polypeptide analog comprising two to fifty units of SEQ ID NO: 5, a polypeptide analog comprising two to ten units of SEQ ID NO: 5, a polypeptide analog

consisting of a sequence of from two to fourteen amino acid units wherein the amino acid units are selected from the group of amino acid units of SEQ ID NO: 5 consisting of glutamic acid (Glu), tryptophan (Trp), glutamine (Gln), threonine (Thr), aspartic acid (Asp), asparagine (Asn), cysteine (Cys), or tyrosine (Tyr), a polypeptide analog having at least 90 % of its amino acid sequence identical to the amino acid sequence set forth in SEQ ID NO: 5, a polypeptide analog having at least 70 % of its amino acid sequence identical to the amino acid sequence set forth in SEQ ID NO: 5, and a polypeptide analog having at least 50 % of its amino acid sequence identical to the amino acid sequence set forth in SEQ ID NO: 5 and mixture(s) thereof, for inhibiting the growth of a tumor or more precisely for inhibiting the growth of prostatic adenocarcinoma, stomach cancer, breast cancer, endometrial, ovarian or other cancers of epithelial secretion, or benign prostate hyperplasia (BPH).

In one embodiment of the second aspect of the present invention, the polypeptide or polypeptide analog may be used with an anticancer drug, such as, for example, mitomycin, idarubicin, cisplatin, 5-fluoro-uracil, methotrexate, adriamycin, daunomycin, taxol (i.e., paclitaxel), taxol derivative (e.g., docetaxel, taxane), and mixtures thereof.

In an additional embodiment of the second aspect of the present invention, the polypeptide or polypeptide analog may be used with a pharmaceutically acceptable carrier.

In a further embodiment of the second aspect of the present invention the polypeptide or polypeptide analog may be used with a time-release means such as, for example, liposomes and polysaccharides for effecting continual dosing of said polypeptide or polypeptide analog.

In other embodiments of the second aspect of the present invention, the polypeptide or polypeptide analog may be used with an anticancer drug and a pharmaceutically acceptable carrier, with an anticancer drug and a time-release means, with a pharmaceutically acceptable carrier and a time-release means, or with an anticancer drug, a pharmaceutically acceptable and a time-release means. Some examples of an anticancer drug, a pharmaceutically acceptable carrier and a time-release means are described herein.

In a third aspect, the present invention relates to a method for treating a patient with a tumor or more precisely with prostatic

adenocarcinoma, stomach cancer, breast cancer, endometrial, ovarian or other cancers of epithelial secretion, or benign prostate hyperplasia (BPH), the method comprising administering to the patient a pharmaceutical composition comprising a polypeptide or polypeptide analog selected from the group consisting of rHuPSP94 as set forth in SEQ ID NO: 2, the decapeptide as set forth in SEQ ID NO: 3, the polypeptide as set forth in SEQ ID NO: 4 (polypeptide 7-21), the polypeptide as set forth in SEQ ID NO: 5 (PCK3145), and the polypeptide as set forth in SEQ ID NO: 6 (polypeptide 76-94), a polypeptide analog selected from the group consisting of a polypeptide analog of at least five contiguous amino acids of SEQ ID NO: 2, of SEQ ID NO: 3, of SEQ ID NO: 4, of SEQ ID NO: 5, or of SEQ ID NO: 6, a polypeptide analog of at least two contiguous amino acids of SEQ ID NO: 2, of SEQ ID NO: 3, of SEQ ID NO: 4, of SEQ ID NO: 5, or of SEQ ID NO: 6, a polypeptide analog consisting of the amino acid sequence $X_1 W Q X_2 D X_1 C X_1 X_2 C X_2 C X_3 X_1 X_2$ as set forth in SEQ ID NO: 89, wherein X_1 is either glutamic acid (Glu), asparagine (Asn) or aspartic acid (Asp), X_2 is either threonine (Thr) or serine (Ser), and X_3 is either tyrosine (Tyr) or phenylalanine (Phe), a polypeptide analog comprising SEQ ID NO: 5 and having an addition of at least one amino acid to its amino-terminus, wherein said polypeptide analog comprising SEQ ID NO: 5 is selected from the group consisting of SEQ ID NO: 59 to SEQ ID NO: 88, a polypeptide analog comprising SEQ ID NO: 5 and having an addition of at least one amino acid to its carboxy-terminus, wherein said polypeptide analog comprising SEQ ID NO: 5 is selected from the group consisting of SEQ ID NO: 10 to SEQ ID NO: 58, a polypeptide analog comprising two to fifty units of SEQ ID NO: 5, a polypeptide analog comprising two to ten units of SEQ ID NO: 5, a polypeptide analog consisting of a sequence of from two to fourteen amino acid units wherein the amino acid units are selected from the group of amino acid units of SEQ ID NO: 5 consisting of glutamic acid (Glu), tryptophan (Trp), glutamine (Gln), threonine (Thr), aspartic acid (Asp), asparagine (Asn), cysteine (Cys), or tyrosine (Tyr), a polypeptide analog having at least 90 % of its amino acid sequence identical to the amino acid sequence set forth in SEQ ID NO: 5, a polypeptide analog having at least 70 % of its amino acid sequence identical to the amino acid sequence set forth in SEQ ID NO: 5, and a polypeptide analog having at least 50 % of its amino acid sequence identical to the amino acid sequence set forth in SEQ ID NO: 5 and mixtures thereof. The polypeptide analog mentioned herein may be capable of inhibiting the growth of a tumor or more precisely may be capable of inhibiting the growth of prostatic adenocarcinoma, stomach cancer, breast cancer, endometrial, ovarian

or other cancers of epithelial secretion, or benign prostate hyperplasia (BPH).

5 The method for treating a patient as described above may result, for example, in the inhibition (e.g., reduction, control, attenuation, prohibition) of the growth of a tumor(s) in a patient suffering for example from prostatic adenocarcinoma, stomach cancer, breast cancer, endometrial, ovarian or other cancers of epithelial secretion, or benign prostate hyperplasia (BPH). The method
10 described above may be performed, for example, by administering to the patient a pharmaceutical composition comprising a polypeptide, a polypeptide analog, or mixtures thereof of the present invention.

15 In one embodiment of the third aspect of the present invention, the polypeptide or polypeptide analog may be used with an anticancer drug, such as, for example, mitomycin, idarubicin, cisplatin, 5-fluoro-uracil, methotrexate, adriamycin, daunomycin, taxol (i.e., paclitaxel), taxol derivative (e.g., docetaxel, taxane), and mixtures thereof.

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 In an additional embodiment of the third aspect of the present invention, the polypeptide or polypeptide analog may be used with a pharmaceutically acceptable carrier.

25 In a further embodiment of the third aspect of the present invention the polypeptide or polypeptide analog may be used with a time-release means such as for example, liposomes and polysaccharides for effecting continual dosing of said polypeptide or polypeptide analog.

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 It other embodiments of the third aspect of the present invention, the polypeptide or polypeptide analog may be used with an anticancer drug and a pharmaceutically acceptable carrier, with an anticancer drug and a time-release means, with a pharmaceutically
35 acceptable carrier and a time-release means, or with an anticancer drug, a pharmaceutically acceptable and a time-release means. Some examples of an anticancer drug, a pharmaceutically acceptable carrier and a time-release means are described herein.

40 In a fourth aspect, the present invention relates to a method for treating a patient with a tumor or more precisely with prostatic adenocarcinoma, stomach cancer, breast cancer, endometrial, ovarian or other cancers of epithelial secretion, or benign prostate hyperplasia (BPH), the method comprising administering to the patient

a pharmaceutical composition including a vector comprising the nucleotide sequence of SEQ ID NO: 9 and a pharmaceutically acceptable carrier or a pharmaceutical composition comprising a polynucleotide selected from the group consisting of a polynucleotide having at least 10 to 285 contiguous residues of SEQ ID NO: 9, and a polynucleotide having at least 10 to 50 contiguous residues of SEQ ID NO: 9, and a pharmaceutically acceptable carrier.

In one embodiment of the fourth aspect of the present invention, the vector or the polynucleotide may be used with an anticancer drug such as, for example, mitomycin, idarubicin, cisplatin, 5-fluoro-uracil, methotrexate, adriamycin, daunomycin, taxol (i.e., paclitaxel), taxol derivative (e.g., docetaxel, taxane), and mixtures thereof.

In an additional embodiment of the fourth aspect of the present invention, the vector or the polynucleotide may be used with a time-release means such as, for example, liposomes and polysaccharides for effecting continual dosing of said vector.

In further embodiment of the fourth aspect of the present invention, the vector or the polynucleotide may be used with an anticancer drug such as, for example, mitomycin, idarubicin, cisplatin, 5-fluoro-uracil, methotrexate, adriamycin, daunomycin, taxol (i.e., paclitaxel), taxol derivative (e.g., docetaxel, taxane), and mixtures thereof and with a time-release means such as, for example, liposomes and polysaccharides for effecting continual dosing of said vector or polynucleotide.

In a fifth aspect, the present invention relates to a pharmaceutical composition for inhibiting (e.g., reducing, controlling, attenuating, prohibiting) the growth of a tumor in a patient suffering from prostatic adenocarcinoma, stomach cancer, breast cancer, endometrial, ovarian or other cancers of epithelial secretion, or benign prostate hyperplasia (BPH), comprising:

a) a polypeptide or a polypeptide analog selected from the group consisting of rHuPSP94 as set forth in SEQ ID NO: 2, the decapeptide as set forth in SEQ ID NO: 3, the polypeptide as set forth in SEQ ID NO: 4 (Polypeptide 7-21), the polypeptide as set forth in SEQ ID NO: 5 (PCK3145), the polypeptide as set forth in SEQ ID NO: 6 (Polypeptide 76-94), a polypeptide analog of at least five contiguous amino acids of SEQ ID NO: 2, of SEQ ID NO: 3,

of SEQ ID NO: 4, of SEQ ID NO: 5, or of SEQ ID NO: 6, a polypeptide analog of at least two contiguous amino acids of SEQ ID NO: 2, of SEQ ID NO: 3, of SEQ ID NO: 4, of SEQ ID NO: 5, or of SEQ ID NO: 6, a polypeptide analog consisting of the amino acid sequence $X_1 W Q X_2 D X_1 C X_1 X_2 C X_2 C X_3 X_1 X_2$ as set forth in SEQ ID NO: 89, wherein X_1 is either glutamic acid (Glu), asparagine (Asn) or aspartic acid (Asp), X_2 is either threonine (Thr) or serine (Ser), and X_3 is either tyrosine (Tyr) or phenylalanine (Phe), a polypeptide analog comprising SEQ ID NO: 5 and having an addition of at least one amino acid to its amino-terminus wherein said polypeptide analog comprising SEQ ID NO: 5 is selected from the group consisting of SEQ ID NO: 59 to SEQ ID NO: 88, a polypeptide analog comprising SEQ ID NO: 5 and having an addition of at least one amino acid to its carboxy-terminus, wherein said polypeptide analog comprising SEQ ID NO: 5 is selected from the group consisting of SEQ ID NO: 10 to SEQ ID NO: 58, a polypeptide analog comprising two to fifty units of SEQ ID NO: 5, a polypeptide analog comprising two to ten units of SEQ ID NO: 5, a polypeptide analog consisting of a sequence of from two to fourteen amino acid units wherein the amino acid units are selected from the group of amino acid units of SEQ ID NO: 5 consisting of glutamic acid (Glu), tryptophan (Trp), glutamine (Gln), threonine (Thr), aspartic acid (Asp), asparagine (Asn), cysteine (Cys), or tyrosine (Tyr), a polypeptide analog having at least 90 % of its amino acid sequence identical to the amino acid sequence set forth in SEQ ID NO: 5, a polypeptide analog having at least 70 % of its amino acid sequence identical to the amino acid sequence set forth in SEQ ID NO: 5, and a polypeptide analog having at least 50 % of its amino acid sequence identical to the amino acid sequence set forth in SEQ ID NO: 5, and mixture(s) thereof, and;

b) an anticancer drug such as, for example, mitomycin, idarubicin, cisplatin, 5-fluoro-uracil, methotrexate, adriamycin, daunomycin, taxol, taxol derivative, and mixtures thereof.

In one embodiment of the fifth aspect of the present invention the pharmaceutical composition may further comprise a time-release means such as, for example, liposomes and polysaccharides for effecting continual dosing of the composition.

In a sixth aspect, the present invention relates to a pharmaceutical composition for inhibiting the growth of a tumor in a patient suffering from prostatic adenocarcinoma, stomach cancer, breast cancer, endometrial, ovarian or other cancers of epithelial secretion, or benign prostate hyperplasia (BPH), comprising:

- a) a polypeptide or polypeptide analog selected from the group consisting of rHuPSP94 as set forth in SEQ ID NO: 2, the decapeptide as set forth in SEQ ID NO: 3, the polypeptide as set forth in SEQ ID NO: 4 (Polypeptide 7-21), the polypeptide as set forth in SEQ ID NO: 5 (PCK3145), the polypeptide as set forth in SEQ ID NO: 6 (Polypeptide 76-94), a polypeptide analog of at least five contiguous amino acids of SEQ ID NO: 2, of SEQ ID NO: 3, of SEQ ID NO: 4, of SEQ ID NO: 5, or of SEQ ID NO: 6, a polypeptide analog of at least two contiguous amino acids of SEQ ID NO: 2, of SEQ ID NO: 3, of SEQ ID NO: 4, of SEQ ID NO: 5, or of SEQ ID NO: 6, a polypeptide analog consisting of the amino acid sequence $X_1 W Q X_2 D X_1 C X_1 X_2 C X_2 C X_3 X_1 X_2$ as set forth in SEQ ID NO: 89, wherein X_1 is either glutamic acid (Glu), asparagine (Asn) or aspartic acid (Asp), X_2 is either threonine (Thr) or serine (Ser), and X_3 is either tyrosine (Tyr) or phenylalanine (Phe), a polypeptide analog comprising SEQ ID NO: 5 and having an addition of at least one amino acid to its amino-terminus wherein said polypeptide analog comprising SEQ ID NO: 5 is selected from the group consisting of SEQ ID NO: 59 to SEQ ID NO: 88, a polypeptide analog comprising SEQ ID NO: 5 and having an addition of at least one amino acid to its carboxy-terminus, wherein said polypeptide analog comprising SEQ ID NO: 5 is selected from the group consisting of SEQ ID NO: 10 to SEQ ID NO: 58, a polypeptide analog comprising two to fifty units of SEQ ID NO: 5, a polypeptide analog comprising two to ten units of SEQ ID NO: 5, a polypeptide analog consisting of a sequence of from two to fourteen amino acid units wherein the amino acid units are selected from the group of amino acid units of SEQ ID NO: 5 consisting of glutamic acid (Glu), tryptophan (Trp), glutamine (Gln), threonine (Thr), aspartic acid (Asp), asparagine (Asn), cysteine (Cys), or tyrosine (Tyr), a polypeptide analog having at least 90 % of its amino acid sequence identical to the amino acid sequence set forth in SEQ ID NO: 5, a

polypeptide analog having at least 70 % of its amino acid sequence identical to the amino acid sequence set forth in SEQ ID NO: 5, and a polypeptide analog having at least 50 % of its amino acid sequence identical to the amino acid sequence set forth in SEQ ID NO: 5, and mixture(s) thereof, and;

b) a pharmaceutically acceptable carrier.

In one embodiment of the sixth aspect of the present invention the pharmaceutical composition may further comprise a time-release means such as, for example, liposomes and polysaccharides for effecting continual dosing of the composition.

In a second embodiment of the sixth aspect of the present invention the pharmaceutical composition may further comprise an anticancer drug such as, for example, mitomycin, idarubicin, cisplatin, 5-fluoro-uracil, methotrexate, adriamycin, daunomycin, taxol, taxol derivative, and mixtures thereof.

In a third embodiment of the sixth aspect of the present invention, the pharmaceutical composition may further comprise a time-release means and an anticancer drug. Examples of time-release means and anticancer drug are described herein.

In a seventh aspect, the present invention relates to a pharmaceutical composition comprising:

a) A polypeptide or polypeptide analog selected from the group consisting of rHuPSP94 as set forth in SEQ ID NO: 2, the decapeptide as set forth in SEQ ID NO: 3, the polypeptide as set forth in SEQ ID NO: 4 (polypeptide 7-21), the polypeptide as set forth in SEQ ID NO: 5 (PCK3145), the polypeptide as set forth in SEQ ID NO: 6 (polypeptide 76-94), a polypeptide analog of at least five contiguous amino acids of SEQ ID NO: 2, of SEQ ID NO: 3, of SEQ ID NO: 4, of SEQ ID NO: 5, or of SEQ ID NO: 6, a polypeptide analog of at least two contiguous amino acids of SEQ ID NO: 2, of SEQ ID NO: 3, of SEQ ID NO: 4, of SEQ ID NO: 5, or of SEQ ID NO: 6, a polypeptide analog consisting of the amino acid sequence $X_1 W Q X_2 D X_1 C X_1 X_2 C X_2 C X_3 X_1 X_2$ as set forth in SEQ ID NO: 89, wherein X_1 is either glutamic acid (Glu), asparagine (Asn) or aspartic acid (Asp), X_2 is either threonine (Thr) or serine (Ser),

and X₃ is either tyrosine (Tyr) or phenylalanine (Phe), a polypeptide analog comprising SEQ ID NO: 5 and having an addition of at least one amino acid to its amino-terminus wherein said polypeptide analog comprising SEQ ID NO: 5 is selected from the group consisting of SEQ ID NO: 59 to SEQ ID NO: 88, a polypeptide analog comprising SEQ ID NO: 5 and having an addition of at least one amino acid to its carboxy-terminus, wherein said polypeptide analog comprising SEQ ID NO: 5 is selected from the group consisting of SEQ ID NO: 10 to SEQ ID NO: 58, a polypeptide analog comprising two to fifty units of SEQ ID NO: 5, a polypeptide analog comprising two to ten units of SEQ ID NO: 5, a polypeptide analog consisting of a sequence of from two to fourteen amino acid units wherein the amino acid units are selected from the group of amino acid units of SEQ ID NO: 5 consisting of glutamic acid (Glu), tryptophan (Trp), glutamine (Gln), threonine (Thr), aspartic acid (Asp), asparagine (Asn), cysteine (Cys), or tyrosine (Tyr), a polypeptide analog having at least 90 % of its amino acid sequence identical to the amino acid sequence set forth in SEQ ID NO: 5, a polypeptide analog having at least 70 % of its amino acid sequence identical to the amino acid sequence set forth in SEQ ID NO: 5, and a polypeptide analog having at least 50 % of its amino acid sequence identical to the amino acid sequence set forth in SEQ ID NO: 5, and mixture(s) thereof, in a therapeutically effective amount, and;

b) an anticancer drug such as, for example, mitomycin, idarubicin, cisplatin, 5-fluoro-uracil, methotrexate, adriamycin, daunomycin, taxol, taxol derivative, and mixtures thereof in a therapeutically effective amount.

In one embodiment of the seventh aspect of the present invention the pharmaceutical composition may further comprise a time-release means such as, for example, liposomes and polysaccharides for effecting continual dosing of the composition.

In an eighth aspect, the present invention relates to a pharmaceutical composition comprising:

a) a polypeptide or polypeptide analog selected from the group consisting of rHuPSP94 as set forth in SEQ ID NO: 2, the decapeptide as set forth in SEQ ID NO: 3, the polypeptide

as set forth in SEQ ID NO: 4 (polypeptide 7-21), the polypeptide as set forth in SEQ ID NO: 5 (PCK3145), the polypeptide as set forth in SEQ ID NO: 6 (polypeptide 76-94), a polypeptide analog of at least five contiguous amino acids of SEQ ID NO: 2, of SEQ ID NO: 3, of SEQ ID NO: 4, of SEQ ID NO: 5, or of SEQ ID NO: 6, a polypeptide analog of at least two contiguous amino acids of SEQ ID NO: 2, of SEQ ID NO: 3, of SEQ ID NO: 4, of SEQ ID NO: 5, or of SEQ ID NO: 6, a polypeptide analog consisting of the amino acid sequence X_1 W Q X_2 D X_1 C X_1 X_2 C X_2 C X_3 X_1 X_2 as set forth in SEQ ID NO: 89, wherein X_1 is either glutamic acid (Glu), asparagine (Asn) or aspartic acid (Asp), X_2 is either threonine (Thr) or serine (Ser), and X_3 is either tyrosine (Tyr) or phenylalanine (Phe), a polypeptide analog comprising SEQ ID NO: 5 and having an addition of at least one amino acid to its amino-terminus wherein said polypeptide analog comprising SEQ ID NO: 5 is selected from the group consisting of SEQ ID NO: 59 to SEQ ID NO: 88, a polypeptide analog comprising SEQ ID NO: 5 and having an addition of at least one amino acid to its carboxy-terminus, wherein said polypeptide analog comprising SEQ ID NO: 5 is selected from the group consisting of SEQ ID NO: 10 to SEQ ID NO: 58, a polypeptide analog comprising two to fifty units of SEQ ID NO: 5, a polypeptide analog comprising two to ten units of SEQ ID NO: 5, a polypeptide analog consisting of a sequence of from two to fourteen amino acid units wherein the amino acid units are selected from the group of amino acid units of SEQ ID NO: 5 consisting of glutamic acid (Glu), tryptophan (Trp), glutamine (Gln), threonine (Thr), aspartic acid (Asp), asparagine (Asn), cysteine (Cys), or tyrosine (Tyr), a polypeptide analog having at least 90 % of its amino acid sequence identical to the amino acid sequence set forth in SEQ ID NO: 5, a polypeptide analog having at least 70 % of its amino acid sequence identical to the amino acid sequence set forth in SEQ ID NO: 5, and a polypeptide analog having at least 50 % of its amino acid sequence identical to the amino acid sequence set forth in SEQ ID NO: 5, and mixture(s) thereof, in a therapeutically effective amount, and;

b) a pharmaceutically acceptable carrier.

In one embodiment of the eighth aspect of the present invention the pharmaceutical composition may further comprise a time-release means such as, for example, liposomes and polysaccharides for effecting continual dosing of the composition.

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In a second embodiment of the eighth aspect of the present invention, the pharmaceutical composition may further comprise an anticancer drug such as, for example, mitomycin, idarubicin, cisplatin, 5-fluoro-uracil, methotrexate, adriamycin, daunomycin, taxol, taxol derivative, and mixtures thereof.

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In a third embodiment of the eighth aspect of the present invention, the pharmaceutical composition may further comprise a time-release means and an anticancer drug. Examples of time-release means and anticancer drug are described herein.

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In a ninth aspect, the present invention relates to a pharmaceutical composition for inhibiting (reducing, controlling, attenuating, prohibiting) the growth of a tumor in a patient suffering from prostatic adenocarcinoma, stomach cancer, breast cancer, endometrial, ovarian or other cancers of epithelial secretion, or benign prostate hyperplasia (BPH), comprising a vector comprising the nucleotide sequence of SEQ ID NO: 9 and a pharmaceutically acceptable carrier, or a polynucleotide selected from the group consisting of a polynucleotide having at least 10 to 285 contiguous residues of SEQ ID NO: 9 and a polynucleotide having at least 10 to 50 contiguous residues of SEQ ID NO: 9, and a pharmaceutically acceptable carrier.

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In one embodiment of the ninth aspect of the present invention, the pharmaceutical composition may further comprise an anticancer drug such as, for example, mitomycin, idarubicin, cisplatin, 5-fluoro-uracil, methotrexate, adriamycin, daunomycin, taxol (i.e., paclitaxel), taxol derivative (e.g., docetaxel, taxane), and mixtures thereof.

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In a tenth aspect, the present invention relates to a pharmaceutical composition for inhibiting the growth of a tumor in a patient, comprising a vector comprising the nucleotide sequence of SEQ ID NO: 9 and a pharmaceutically acceptable carrier, or a polynucleotide selected from the group consisting of a polynucleotide having at least 10 to 285 contiguous residues of SEQ ID NO: 9 and a polynucleotide having at least 10 to 50 contiguous residues of SEQ ID NO: 9, and a pharmaceutically acceptable carrier.

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In one embodiment of the tenth aspect of the present invention, the pharmaceutical composition may further comprise an anticancer drug such as, for example, mitomycin, idarubicin, cisplatin, 5-fluoro-uracil, methotrexate, adriamycin, daunomycin, taxol (i.e.,
5 paclitaxel), taxol derivative (e.g., docetaxel, taxane), and mixtures thereof.

In an eleventh aspect, the present invention relates to a method for treating patients with a disease characterized by elevated
10 levels of FSH comprising administering a pharmaceutical composition in an appropriate dosage form, the pharmaceutical composition comprising a polypeptide or polypeptide analog selected from the group consisting of rHuPSP94 as set forth SEQ ID NO: 2, the
15 decapeptide as set forth in SEQ ID NO: 3, the polypeptide as set forth in SEQ ID NO: 4, the polypeptide as set forth in SEQ ID NO: 5, and the polypeptide as set forth in SEQ ID NO: 6, a polypeptide analog of at least five contiguous amino acids of SEQ ID NO: 2, of
SEQ ID NO: 3, of SEQ ID NO: 4, of SEQ ID NO: 5, or of SEQ ID NO: 6, a
20 polypeptide analog of at least two contiguous amino acids of SEQ ID NO: 2, of SEQ ID NO: 3, of SEQ ID NO: 4, of SEQ ID NO: 5, or of SEQ ID NO: 6, a polypeptide analog consisting of the amino acid sequence
 $X_1 W Q X_2 D X_1 C X_1 X_2 C X_2 C X_3 X_1 X_2$ as set forth in SEQ ID NO: 89,
wherein X_1 is either glutamic acid (Glu), asparagine (Asn) or
aspartic acid (Asp), X_2 is either threonine (Thr) or serine (Ser),
25 and X_3 is either tyrosine (Tyr) or phenylalanine (Phe), a polypeptide analog comprising SEQ ID NO: 5 and having an addition of at least one amino acid to its amino-terminus, wherein said polypeptide analog
comprising SEQ ID NO: 5 is selected from the group consisting of SEQ
ID NO: 59 to SEQ ID NO: 88, a polypeptide analog comprising SEQ ID
30 NO: 5 and having an addition of at least one amino acid to its carboxy-terminus, wherein said polypeptide analog comprising SEQ ID NO: 5 is selected from the group consisting of SEQ ID NO: 10 to SEQ ID
NO: 58, a polypeptide analog comprising two to fifty units of SEQ ID
NO: 5, a polypeptide analog comprising two to ten units of SEQ ID NO:
35 5, a polypeptide analog consisting of a sequence of from two to fourteen amino acid units wherein the amino acid units are selected from the group of amino acid units of SEQ ID NO: 5 consisting of
glutamic acid (Glu), tryptophan (Trp), glutamine (Gln), threonine (Thr), aspartic acid (Asp), asparagine (Asn), cysteine (Cys), or
40 tyrosine (Tyr), a polypeptide analog having at least 90 % of its amino acid sequence identical to the amino acid sequence set forth in
SEQ ID NO: 5, a polypeptide analog having at least 70 % of its amino acid sequence identical to the amino acid sequence set forth in SEQ
ID NO: 5, and a polypeptide analog having at least 50 % of its amino

acid sequence identical to the amino acid sequence set forth in SEQ ID NO: 5, and mixtures thereof, and a pharmaceutically acceptable carrier in a human dose.

5 In a twelfth aspect, the present invention relates to the use of a polypeptide or a polypeptide analog selected from the group consisting of rHuPSP94 as set forth in SEQ ID NO: 2, the decapeptide as set forth in SEQ ID NO: 3, the polypeptide as set forth in SEQ ID NO: 4 (polypeptide 7-21), the polypeptide as set forth in SEQ ID NO: 5 (PCK3145), and the polypeptide as set forth in SEQ ID NO: 6 (polypeptide 76-94), a polypeptide analog of at least five contiguous amino acids of SEQ ID NO: 2, of SEQ ID NO: 3, of SEQ ID NO: 4, of SEQ ID NO: 5, or of SEQ ID NO: 6, a polypeptide analog of at least two contiguous amino acids of SEQ ID NO: 2, of SEQ ID NO: 3, of SEQ ID NO: 4, of SEQ ID NO: 5, or of SEQ ID NO: 6, a polypeptide analog consisting of the amino acid sequence $X_1 W Q X_2 D X_1 C X_1 X_2 C X_2 C X_3 X_1 X_2$ as set forth in SEQ ID NO: 89, wherein X_1 is either glutamic acid (Glu), asparagine (Asn) or aspartic acid (Asp), X_2 is either threonine (Thr) or serine (Ser), and X_3 is either tyrosine (Tyr) or phenylalanine (Phe), a polypeptide analog comprising SEQ ID NO: 5 and having an addition of at least one amino acid to its amino-terminus, wherein said polypeptide analog comprising SEQ ID NO: 5 is selected from the group consisting of SEQ ID NO: 59 to SEQ ID NO: 88, a polypeptide analog comprising SEQ ID NO: 5 and having an addition of at least one amino acid to its carboxy-terminus, wherein said polypeptide analog comprising SEQ ID NO: 5 is selected from the group consisting of SEQ ID NO: 10 to SEQ ID NO: 58, a polypeptide analog comprising two to fifty units of SEQ ID NO: 5, a polypeptide analog comprising two to ten units of SEQ ID NO: 5, a polypeptide analog consisting of a sequence of from two to fourteen amino acid units wherein the amino acid units are selected from the group of amino acid units of SEQ ID NO: 5 consisting of glutamic acid (Glu), tryptophan (Trp), glutamine (Gln), threonine (Thr), aspartic acid (Asp), asparagine (Asn), cysteine (Cys), or tyrosine (Tyr), a polypeptide analog having at least 90 % of its amino acid sequence identical to the amino acid sequence set forth in SEQ ID NO: 5, a polypeptide analog having at least 70 % of its amino acid sequence identical to the amino acid sequence set forth in SEQ ID NO: 5, and a polypeptide analog having at least 50 % of its amino acid sequence identical to the amino acid sequence set forth in SEQ ID NO: 5 and mixture(s) thereof, for treating patients with a disease characterized by elevated levels of FSH.

The use of a polypeptide or a polypeptide analog selected from the group consisting of rHuPSP94 as set forth in SEQ ID NO: 2, the decapeptide as set forth in SEQ ID NO: 3, the polypeptide as set forth in SEQ ID NO: 4 (polypeptide 7-21), the polypeptide as set forth in SEQ ID NO: 5 (PCK3145), the polypeptide as set forth in SEQ ID NO: 6 (polypeptide 76-94), a polypeptide analog of at least five contiguous amino acids of SEQ ID NO: 2, of SEQ ID NO: 3, of SEQ ID NO: 4, of SEQ ID NO: 5, or of SEQ ID NO: 6, a polypeptide analog of at least two contiguous amino acids of SEQ ID NO: 2, of SEQ ID NO: 3, of SEQ ID NO: 4, of SEQ ID NO: 5, or of SEQ ID NO: 6, a polypeptide analog consisting of the amino acid sequence $X_1 W Q X_2 D X_1 C X_1 X_2 C X_2 C X_3 X_1 X_2$ as set forth in SEQ ID NO: 89, wherein X_1 is either glutamic acid (Glu), asparagine (Asn) or aspartic acid (Asp), X_2 is either threonine (Thr) or serine (Ser), and X_3 is either tyrosine (Tyr) or phenylalanine (Phe), a polypeptide analog comprising SEQ ID NO: 5 and having an addition of at least one amino acid to its amino-terminus, wherein said polypeptide analog comprising SEQ ID NO: 5 is selected from the group consisting of SEQ ID NO: 59 to SEQ ID NO: 88, a polypeptide analog comprising SEQ ID NO: 5 and having an addition of at least one amino acid to its carboxy-terminus, wherein said polypeptide analog comprising SEQ ID NO: 5 is selected from the group consisting of SEQ ID NO: 10 to SEQ ID NO: 58, a polypeptide analog comprising two to fifty units of SEQ ID NO: 5, a polypeptide analog comprising two to ten units of SEQ ID NO: 5, a polypeptide analog consisting of a sequence of from two to fourteen amino acid units wherein the amino acid units are selected from the group of amino acid units of SEQ ID NO: 5 consisting of glutamic acid (Glu), tryptophan (Trp), glutamine (Gln), threonine (Thr), aspartic acid (Asp), asparagine (Asn), cysteine (Cys), or tyrosine (Tyr), a polypeptide analog having at least 90 % of its amino acid sequence identical to the amino acid sequence set forth in SEQ ID NO: 5, a polypeptide analog having at least 70 % of its amino acid sequence identical to the amino acid sequence set forth in SEQ ID NO: 5, and a polypeptide analog having at least 50 % of its amino acid sequence identical to the amino acid sequence set forth in SEQ ID NO: 5 and mixtures thereof for the manufacture of a medicament for the therapeutic treatment of prostatic adenocarcinoma, stomach cancer, breast cancer, endometrial, ovarian or other cancers of epithelial secretion, benign prostate hyperplasia (BPH) or a disease characterized by elevated levels of FSH.

In accordance with the present invention, rHuPSP94 may be used in a dosage range from about 10 micrograms/kg/day to about 4 milligrams/kg/day, in a dosage range from about 500 picograms/kg/day

to about 1 milligram/kg/day, in a dosage range from about 5 nanograms/kg/day to about 10 micrograms/kg/day or in a dosage range from about 5 nanograms/kg/day to about 500 nanograms/kg/day.

5 In accordance with the present invention, the decapeptide as set forth in SEQ ID NO: 3, the polypeptide as set forth in SEQ ID NO: 4, the polypeptide as set forth in SEQ ID NO: 5, the polypeptide as set forth in SEQ ID NO: 6, and mixtures thereof may be used in a dosage range from about 100 nanograms/kg/day to about 4
10 milligrams/kg/day.

In accordance with the present invention, the anticancer drug may be mixed or not with a polypeptide or polypeptide analog or mixtures thereof or it may be given separately, by a different route,
15 or even in a different administration schedule (e.g., a different time or day).

In accordance with the present invention administration of the composition may be performed by any suitable routes including
20 administration by injection via the intra-muscular (IM), subcutaneous (SC), intra-dermal (ID), intra-venous (IV) or intra-peritoneal (IP) routes or administration at the mucosal membranes including the oral and nasal cavity membranes using any suitable means.

25 In accordance with the present invention, the composition may be used to treat gastrointestinal cancer.

It is known in the art that the proteins or polypeptides of the present invention may be made according to methods present in the
30 art. The polypeptides of the present invention may be prepared for example, from bacterial cell extracts, or through the use of recombinant techniques. Polypeptides of the present invention may, for example, be produced by transformation (transfection, transduction, or infection) of a host cell with all or part of a
35 rHuPSP94 (SEQ ID NO: 2), the decapeptide as set forth in SEQ ID NO: 3, the polypeptide as set forth in SEQ ID NO: 4 (polypeptide 7-21), the polypeptide as set forth in SEQ ID NO: 5 (PCK3145), and the polypeptide as set forth in SEQ ID NO: 6 (polypeptide 76-94) encoding DNA sequence in a suitable expression vehicle. Examples of suitable
40 expression vehicles comprise for example, plasmids, viral particles, artificial chromosomes and phages. The entire expression vehicle, or a part thereof, may be integrated into the host cell genome. In some circumstances, it is desirable to employ an inducible expression vector.

Any of a wide variety of expression systems may be used to provide the recombinant protein. The precise host cell used is not critical to the invention. Polypeptides of the present invention may be produced in a prokaryotic host (e.g., *E. coli* or *B. subtilis*) or in a eukaryotic host (yeast e.g., *Saccharomyces* or *Pichia Pastoris*; mammalian cells, e.g., monkey COS cells, mouse 3T3 cells (Todaro GJ and Green H., *J. Cell Biol.* 17: 299-313, 1963), Chinese Hamster Ovary cells (CHO) (Puck TT et al., *J. Exp. Med.* 108: 945-956, 1958), BHK, human kidney 293 cells (ATCC: CRL-1573), or human HeLa cells (ATCC: CCL-2); or insect cells).

In a yeast cell expression system such as *Pichia Pastoris* (*P. Pastoris*), DNA sequence encoding polypeptides of the present invention may be cloned into a suitable expression vector such as the pPIC9 vector (Invitrogen). Upon introduction of a vector containing the DNA sequence encoding all or part of the polypeptides of the present invention into the *P. Pastoris* host cells, recombination event may occur for example in the AOX1 locus. Such recombination event may place the DNA sequence of the various polypeptides of the present invention under the dependency of the AOX1 gene promoter. Successful insertion of a gene (DNA sequence) encoding polypeptides of the present invention may result in an expression of such polypeptides that is regulated and/or induced by methanol added in the growth media of the host cell (for reference see Buckholz, R.G. and Gleeson, M.A.G., *Biotechnology*, 9:1067-1072, 1991; Cregg, J.M., et al., *Biotechnology*, 11:905-910, 1993; Sreekrishna, K., et al., *J. Basic Microbiol.*, 28:265-278, 1988; Wegner, G.H., *FEMS Microbiology Reviews*, 87:279-284, 1990).

In mammalian host cells, a number of viral-based expression systems may be utilized. For example, in the event where an adenovirus is used as an expression vector for the polypeptides of the present invention, nucleic acid sequence may be ligated to an adenovirus transcription/translation control complex (e.g., the late promoter and tripartite leader sequence). This chimeric gene may be inserted into the adenovirus genome, for example, by in vitro or in vivo recombination. Insertion into a non-essential region of the viral genome (e.g., region E1 or E3) may result in a recombinant virus that is viable and capable of expressing polypeptides of the present invention in infected hosts.

Proteins and polypeptides of the present invention may also be produced by plant cells. Expression vectors such as cauliflower

mosaic virus and tobacco mosaic virus and plasmid expression vectors (e.g., Ti plasmid) may be used for the expression of polypeptides in plant cells. Such cells are available from a wide range of sources (e.g., the American Type Culture Collection, Rockland, Md.). The methods of transformation or transfection and the choice of expression vehicle are of course to be chosen accordingly to the host cell selected.

In an insect cell expression system such as *Autographa californica* nuclear polyhedrosis virus (AcNPV), which grows in *Spodoptera frugiperda* cells, AcNPV may be used as a vector to express foreign genes. For example, DNA sequence coding for all or part of the polypeptides of the present invention may be cloned into non-essential regions of the virus (for example the polyhedrin gene) and placed under control of an AcNPV promoter, (e.g., the polyhedrin promoter). Successful insertion of a gene (i.e., DNA sequence) encoding polypeptides of the present invention may result in inactivation of the polyhedrin gene and production of non-occluded recombinant virus (i.e., virus lacking the proteinaceous coat encoded by the polyhedrin gene). These recombinant viruses may be used to infect *spodoptera frugiperda* cells in which the inserted gene is expressed.

In addition, a host cell may be chosen for its ability to modulate the expression of the inserted sequences, or to modify or process the gene product in a specific, desired fashion. Such modifications (e.g., glycosylation) and processing (e.g., cleavage) of protein products may be important for the function of the protein. Different host cells have characteristics and specific mechanisms for posttranslational processing and modification of proteins and gene products. Of course, cell lines or host systems may be chosen to ensure desired modification and processing of the foreign protein expressed. To this end, eukaryotic host cells that possess the cellular machinery for proper processing of the primary transcript, glycosylation, and phosphorylation of the gene product may be used. Such mammalian host cells comprise for example, but are not limited to, CHO, VERO, BHK, HeLa, COS, MDCK, 293, and 3T3.

Alternatively, polypeptides of the present invention may be produced by a stably transfected mammalian cell line. A number of vectors suitable for stable transfection of mammalian cells are available to the public; methods for constructing such cell lines are also publicly available. In one example, cDNA encoding the rHuPSP94 protein may be cloned into an expression vector that includes the

dihydrofolate reductase (DHFR) gene. Integration of the plasmid and, therefore, DNA sequence of polypeptides of the present invention, into the host cell chromosome may be selected for by including methotrexate in the cell culture media. This selection may be
5 accomplished in most cell types.

Specific initiation signals may also be required for the efficient translation of DNA sequences inserted in a suitable expression vehicle as described above. These signals may include the
10 ATG initiation codon and adjacent sequences. For example, in the event where gene or cDNA encoding polypeptides of the present invention, would not have their own initiation codon and adjacent sequences, additional translational control signals may be needed. For example, exogenous translational control signals, including,
15 perhaps, the ATG initiation codon, may be needed. It is known in the art that the initiation codon must be in phase with the reading frame of the polypeptide sequence to ensure proper translation of the desired polypeptide. Exogenous translational control signals and initiation codons may be of a variety of origins, including both
20 natural and synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements, transcription terminators.

As may be appreciated, a number of modifications may be made to
25 the polypeptides and fragments of the present invention without deleteriously affecting the biological activity of the polypeptides or fragments. Polypeptides of the present invention comprises for example, those containing amino acid sequences modified either by natural processes, such as posttranslational processing, or by
30 chemical modification techniques which are known in the art. Modifications may occur anywhere in a polypeptide including the polypeptide backbone, the amino acid side-chains and the amino or carboxy termini. It will be appreciated that the same type of modification may be present in the same or varying degrees at several
35 sites in a given polypeptide. Also, a given polypeptide may contain many types of modifications. Polypeptides may be branched as a result of ubiquitination, and they may be cyclic, with or without branching. Cyclic, branched and branched cyclic polypeptides may result from posttranslational natural processes or may be made by
40 synthetic methods. Modifications comprise for example, without limitation, acetylation, acylation, addition of acetamidomethyl (Acm) group, ADP-ribosylation, amidation, covalent attachment to flavin, covalent attachment to a heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of a lipid

or lipid derivative, covalent attachment of phosphatidylinositol, cross-linking, cyclization, disulfide bond formation, demethylation, formation of covalent cross-links, formation of cystine, formation of pyroglutamate, formylation, gamma-carboxylation, glycosylation, GPI anchor formation, hydroxylation, iodination, methylation, myristoylation, oxidation, proteolytic processing, phosphorylation, prenylation, racemization, selenoylation, sulfation, transfer-RNA mediated addition of amino acids to proteins such as arginylation and ubiquitination (for reference see, Protein-structure and molecular properties, 2nd Ed., T.E. Creighton, W.H. Freeman and Company, New-York, 1993).

Other type of polypeptide modification may comprises for example, amino acid insertion (i.e., addition), deletion and substitution (i.e., replacement), either conservative or non-conservative (e.g., D-amino acids, desamino acids) in the polypeptide sequence where such changes do not substantially alter the overall biological activity of the polypeptide. Polypeptides of the present invention comprise for example, biologically active mutants, variants, fragments, chimeras, and analogs; fragments encompass amino acid sequences having truncations of one or more amino acids, wherein the truncation may originate from the amino terminus (N-terminus), carboxy terminus (C-terminus), or from the interior of the protein. Analogs of the invention involve an insertion or a substitution of one or more amino acids. Variants, mutants, fragments, chimeras and analogs may have the biological property of polypeptides of the present invention which is to inhibit growth of prostatic adenocarcinoma, stomach cancer, breast cancer, endometrial, ovarian or other cancers of epithelial secretion, or benign prostate hyperplasia (BPH).

Example of substitutions may be those, which are conservative (i.e., wherein a residue is replaced by another of the same general type). As is understood, naturally occurring amino acids may be sub-classified as acidic, basic, neutral and polar, or neutral and non-polar. Furthermore, three of the encoded amino acids are aromatic. It may be of use that encoded polypeptides differing from the determined polypeptide of the present invention contain substituted codons for amino acids, which are from the same group as that of the amino acid be replaced. Thus, in some cases, the basic amino acids Lys, Arg and His may be interchangeable; the acidic amino acids Asp and Glu may be interchangeable; the neutral polar amino acids Ser, Thr, Cys, Gln, and Asn may be interchangeable; the non-polar aliphatic amino acids Gly, Ala, Val, Ile, and Leu are interchangeable

but because of size Gly and Ala are more closely related and Val, Ile and Leu are more closely related to each other, and the aromatic amino acids Phe, Trp and Tyr may be interchangeable.

5 It should be further noted that if the polypeptides are made synthetically, substitutions by amino acids, which are not naturally encoded by DNA may also be made. For example, alternative residues include the omega amino acids of the formula $\text{NH}_2(\text{CH}_2)_n\text{COOH}$ wherein n is 2-6. These are neutral nonpolar amino acids, as are sarcosine, t-
10 butyl alanine, t-butyl glycine, N-methyl isoleucine, and norleucine. Phenylglycine may substitute for Trp, Tyr or Phe; citrulline and methionine sulfoxide are neutral nonpolar, cysteic acid is acidic, and ornithine is basic. Proline may be substituted with
15 hydroxyproline and retain the conformation conferring properties.

 It is known in the art that mutants or variants may be generated by substitutional mutagenesis and retain the biological activity of the polypeptides of the present invention. These variants have at least one amino acid residue in the protein molecule
20 removed and a different residue inserted in its place. For example, one site of interest for substitutional mutagenesis may include but are not restricted to sites identified as the active site(s), or immunological site(s). Other sites of interest may be those, for example, in which particular residues obtained from various species
25 are identical. These positions may be important for biological activity. Examples of substitutions identified as "conservative substitutions" are shown in table 1. If such substitutions result in a change not desired, then other type of substitutions, denominated
30 "exemplary substitutions" in table 1, or as further described herein in reference to amino acid classes, are introduced and the products screened.

 In some cases it may be of interest to modify the biological activity of a polypeptide by amino acid substitution, insertion, or
35 deletion. For example, modification of a polypeptide may result in an increase in the polypeptide's biological activity, may modulate its toxicity, may result in changes in bioavailability or in stability, or may modulate its immunological activity or immunological identity. Substantial modifications in function or
40 immunological identity are accomplished by selecting substitutions that differ significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation. (b) the charge or hydrophobicity of the molecule at the target site, or

(c) the bulk of the side chain. Naturally occurring residues are divided into groups based on common side chain properties:

- 5 (1) hydrophobic: norleucine, methionine (Met), Alanine (Ala),
Valine (Val), Leucine (Leu), Isoleucine (Ile)
- (2) neutral hydrophilic: Cysteine (Cys), Serine (Ser), Threonine
(Thr)
- (3) acidic: Aspartic acid (Asp), Glutamic acid (Glu)
- 10 (4) basic: Asparagine (Asn), Glutamine (Gln), Histidine (His),
Lysine (Lys), Arginine (Arg)
- (5) residues that influence chain orientation: Glycine (Gly),
Proline (Pro); and
- (6) aromatic: Tryptophan (Trp), Tyrosine (Tyr), Phenylalanine
(Phe)

15

Non-conservative substitutions will entail exchanging a member of one of these classes for another.

TABLE 1. Preferred amino acid substitution

Original residue	Exemplary substitution	Conservative substitution
Ala (A)	Val, Leu, Ile	Val
Arg (R)	Lys, Gln, Asn	Lys
Asn (N)	Gln, His, Lys, Arg	Gln
Asp (D)	Glu	Glu
Cys (C)	Ser	Ser
Gln (Q)	Asn	Asn
Glu (E)	Asp	Asp
Gly (G)	Pro	Pro
His (H)	Asn, Gln, Lys, Arg	Arg
Ile (I)	Leu, Val, Met, Ala, Phe, norleucine	Leu
Leu (L)	Norleucine, Ile, Val, Met, Ala, Phe	Ile
Lys (K)	Arg, Gln, Asn	Arg
Met (M)	Leu, Phe, Ile	Leu
Phe (F)	Leu, Val, Ile, Ala	Leu
Pro (P)	Gly	Gly
Ser (S)	Thr	Thr
Thr (T)	Ser	Ser
Trp (W)	Tyr	Tyr
Tyr (Y)	Trp, Phe, Thr, Ser	Phe
Val (V)	Ile, Leu, Met, Phe, Ala, norleucine	Leu

5

Example of analogs of PCK3145 (SEQ ID NO: 5) exemplified by amino acid substitutions has been illustrated below.

Position	1				5					10					15
PCK3145	E	W	Q	T	D	N	C	E	T	C	T	C	Y	E	T
SEQ ID NO: 89	X ₁	W	Q	X ₂	D	X ₁	C	X ₁	X ₂	C	X ₂	C	X ₃	X ₁	X ₂

10

For example, X₁ could be glutamic acid (i.e., glutamate) (Glu), aspartic acid (aspartate) (Asp), or asparagine (Asn), X₂ could be threonine (Thr) or serine (Ser) and X₃ could be tyrosine (Tyr) or phenylalanine (Phe).

Amino acids sequence insertions (e.g., additions) include amino and/or carboxyl-terminal fusions ranging in length from one residues to polypeptides containing a hundred or more residues, as well as intrasequence insertions of single or multiple amino acid residues.

5 Other insertional variants include the fusion of the N- or C-terminus of the protein to a homologous or heterologous polypeptide forming a chimera. Chimeric polypeptides (i.e., chimeras, polypeptide analog) comprise sequence of the polypeptides of the present invention fused to homologous or heterologous sequence. Said homologous or
10 heterologous sequence encompass those which, when formed into a chimera with the polypeptides of the present invention retain one or more biological or immunological properties. Examples of homologous sequences fused to PCK3145 (SEQ ID NO: 5) are illustrated below (1 to 79). Such homologous sequences are derived as it is the case for
15 PCK3145, from rHuPSP94 (SEQ ID NO: 2).

- 1) EWQTDNCETCTCYETE (SEQ ID NO: 10)
- 2) EWQTDNCETCTCYETEI (SEQ ID NO: 11)
- 3) EWQTDNCETCTCYETEIS (SEQ ID NO: 12)
- 20 4) EWQTDNCETCTCYETEISC (SEQ ID NO: 13)
- 5) EWQTDNCETCTCYETEISCC (SEQ ID NO: 14)
- 6) EWQTDNCETCTCYETEISCCT (SEQ ID NO: 15)
- 7) EWQTDNCETCTCYETEISCCTL (SEQ ID NO: 16)
- 8) EWQTDNCETCTCYETEISCCTLV (SEQ ID NO: 17)
- 25 9) EWQTDNCETCTCYETEISCCTLVS (SEQ ID NO: 18)
- 10) EWQTDNCETCTCYETEISCCTLVST (SEQ ID NO: 19)
- 11) EWQTDNCETCTCYETEISCCTLVSTP (SEQ ID NO: 20)
- 12) EWQTDNCETCTCYETEISCCTLVSTPV (SEQ ID NO: 21)
- 13) EWQTDNCETCTCYETEISCCTLVSTPVG (SEQ ID NO: 22)
- 30 14) EWQTDNCETCTCYETEISCCTLVSTPVGY (SEQ ID NO: 23)
- 15) EWQTDNCETCTCYETEISCCTLVSTPVGYD (SEQ ID NO: 24)
- 16) EWQTDNCETCTCYETEISCCTLVSTPVGYDK (SEQ ID NO: 25)
- 17) EWQTDNCETCTCYETEISCCTLVSTPVGYDKD (SEQ ID NO: 26)
- 18) EWQTDNCETCTCYETEISCCTLVSTPVGYDKDN (SEQ ID NO: 27)
- 35 19) EWQTDNCETCTCYETEISCCTLVSTPVGYDKDNC (SEQ ID NO: 28)
- 20) EWQTDNCETCTCYETEISCCTLVSTPVGYDKDNCQ (SEQ ID NO: 29)
- 21) EWQTDNCETCTCYETEISCCTLVSTPVGYDKDNCQR (SEQ ID NO: 30)
- 22) EWQTDNCETCTCYETEISCCTLVSTPVGYDKDNCQRI (SEQ ID NO: 31)
- 23) EWQTDNCETCTCYETEISCCTLVSTPVGYDKDNCQRIF (SEQ ID NO: 32)
- 40 24) EWQTDNCETCTCYETEISCCTLVSTPVGYDKDNCQRIFK (SEQ ID NO: 33)

- 25) EWQTDNCETCTCYETEISCCTLVSTPVG YDKDNCQRIFKK (SEQ ID NO: 34)
- 26) EWQTDNCETCTCYETEISCCTLVSTPVG YDKDNCQRIFKKE (SEQ ID NO: 35)
- 27) EWQTDNCETCTCYETEISCCTLVSTPVG YDKDNCQRIFKKED (SEQ ID NO: 36)
- 28) EWQTDNCETCTCYETEISCCTLVSTPVG YDKDNCQRIFKKEDC (SEQ ID NO: 37)
- 5 29) EWQTDNCETCTCYETEISCCTLVSTPVG YDKDNCQRIFKKEDCK (SEQ ID NO: 38)
- 30) EWQTDNCETCTCYETEISCCTLVSTPVG YDKDNCQRIFKKEDCKY (SEQ ID NO: 39)
- 31) EWQTDNCETCTCYETEISCCTLVSTPVG YDKDNCQRIFKKEDCKYI (SEQ ID NO: 40)
- 32) EWQTDNCETCTCYETEISCCTLVSTPVG YDKDNCQRIFKKEDCKYIV
(SEQ ID NO: 41)
- 10 33) EWQTDNCETCTCYETEISCCTLVSTPVG YDKDNCQRIFKKEDCKYIVV
(SEQ ID NO: 42)
- 34) EWQTDNCETCTCYETEISCCTLVSTPVG YDKDNCQRIFKKEDCKYIVVE
(SEQ ID NO: 43)
- 35) EWQTDNCETCTCYETEISCCTLVSTPVG YDKDNCQRIFKKEDCKYIVVEK
(SEQ ID NO: 44)
- 15 36) EWQTDNCETCTCYETEISCCTLVSTPVG YDKDNCQRIFKKEDCKYIVVEKK
(SEQ ID NO: 45)
- 37) EWQTDNCETCTCYETEISCCTLVSTPVG YDKDNCQRIFKKEDCKYIVVEKKD
(SEQ ID NO: 46)
- 20 38) EWQTDNCETCTCYETEISCCTLVSTPVG YDKDNCQRIFKKEDCKYIVVEKKDP
(SEQ ID NO: 47)
- 39) EWQTDNCETCTCYETEISCCTLVSTPVG YDKDNCQRIFKKEDCKYIVVEKKDPK
(SEQ ID NO: 48)
- 40) EWQTDNCETCTCYETEISCCTLVSTPVG YDKDNCQRIFKKEDCKYIVVEKKDPKK
(SEQ ID NO: 49)
- 25 41) EWQTDNCETCTCYETEISCCTLVSTPVG YDKDNCQRIFKKEDCKYIVVEKKDPKKT
(SEQ ID NO: 50)
- 42) EWQTDNCETCTCYETEISCCTLVSTPVG YDKDNCQRIFKKEDCKYIVVEKKDPKKT
(SEQ ID NO: 51)
- 30 43) EWQTDNCETCTCYETEISCCTLVSTPVG YDKDNCQRIFKKEDCKYIVVEKKDPKKTCS
(SEQ ID NO: 52)
- 44) EWQTDNCETCTCYETEISCCTLVSTPVG YDKDNCQRIFKKEDCKYIVVEKKDPKKT
CSV (SEQ ID NO: 53)
- 35 45) EWQTDNCETCTCYETEISCCTLVSTPVG YDKDNCQRIFKKEDCKYIVVEKKDPKKT
CSV (SEQ ID NO: 54)
- 46) EWQTDNCETCTCYETEISCCTLVSTPVG YDKDNCQRIFKKEDCKYIVVEKKDPKKT
CSVSE (SEQ ID NO: 55)
- 47) EWQTDNCETCTCYETEISCCTLVSTPVG YDKDNCQRIFKKEDCKYIVVEKKDPKKT
CSVSEW (SEQ ID NO: 56)

- 48) EWQTDNCETCTCYETEISCCTLVSTPVG YDKDNCQRIFKKEDCKYIVVEKKDPKKT
CSVSEWI (SEQ ID NO: 57)
- 49) EWQTDNCETCTCYETEISCCTLVSTPVG YDKDNCQRIFKKEDCKYIVVEKKDPKKT
CSVSEWII (SEQ ID NO: 58)
- 5 50) SCYFIPNEGVP GDSTRKCMDLKG NKHPINSEWQTDNCETCTCYET (SEQ ID NO: 88)
- 51) CYFIPNEGVP GDSTRKCMDLKG NKHPINSEWQTDNCETCTCYET (SEQ ID NO: 87)
- 52) YFIPNEGVP GDSTRKCMDLKG NKHPINSEWQTDNCETCTCYET (SEQ ID NO: 86)
- 53) FIPNEGVP GDSTRKCMDLKG NKHPINSEWQTDNCETCTCYET (SEQ ID NO: 85)
- 54) IPNEGVP GDSTRKCMDLKG NKHPINSEWQTDNCETCTCYET (SEQ ID NO: 84)
- 10 55) PNEGVP GDSTRKCMDLKG NKHPINSEWQTDNCETCTCYET (SEQ ID NO: 83)
- 56) NEGVP GDSTRKCMDLKG NKHPINSEWQTDNCETCTCYET (SEQ ID NO: 82)
- 57) EGVPGDSTRKCMDLKG NKHPINSEWQTDNCETCTCYET (SEQ ID NO: 81)
- 58) GVPGDSTRKCMDLKG NKHPINSEWQTDNCETCTCYET (SEQ ID NO: 80)
- 59) VPGDSTRKCMDLKG NKHPINSEWQTDNCETCTCYET (SEQ ID NO: 79)
- 15 60) PGDSTRKCMDLKG NKHPINSEWQTDNCETCTCYET (SEQ ID NO: 78)
- 61) GDSTRKCMDLKG NKHPINSEWQTDNCETCTCYET (SEQ ID NO: 77)
- 62) DSTRKCMDLKG NKHPINSEWQTDNCETCTCYET (SEQ ID NO: 76)
- 63) STRKCMDLKG NKHPINSEWQTDNCETCTCYET (SEQ ID NO: 75)
- 64) TRKCMDLKG NKHPINSEWQTDNCETCTCYET (SEQ ID NO: 74)
- 20 65) RKCMDLKG NKHPINSEWQTDNCETCTCYET (SEQ ID NO: 73)
- 66) KCMDLKG NKHPINSEWQTDNCETCTCYET (SEQ ID NO: 72)
- 67) CMDLKG NKHPINSEWQTDNCETCTCYET (SEQ ID NO: 71)
- 68) MDLKG NKHPINSEWQTDNCETCTCYET (SEQ ID NO: 70)
- 69) DLKG NKHPINSEWQTDNCETCTCYET (SEQ ID NO: 69)
- 25 70) LKG NKHPINSEWQTDNCETCTCYET (SEQ ID NO: 68)
- 71) KG NKHPINSEWQTDNCETCTCYET (SEQ ID NO: 67)
- 72) G NKHPINSEWQTDNCETCTCYET (SEQ ID NO: 66)
- 73) N NKHPINSEWQTDNCETCTCYET (SEQ ID NO: 65)
- 74) KHPINSEWQTDNCETCTCYET (SEQ ID NO: 64)
- 30 75) HPINSEWQTDNCETCTCYET (SEQ ID NO: 63)
- 76) PINSEWQTDNCETCTCYET (SEQ ID NO: 62)
- 77) INSEWQTDNCETCTCYET (SEQ ID NO: 61)
- 78) NSEWQTDNCETCTCYET (SEQ ID NO: 60)
- 79) SEWQTDNCETCTCYET (SEQ ID NO: 59)
- 35

Other type of chimera generated by homologous fusion includes new polypeptides formed by the repetition of two or more polypeptides of the present invention. The number of repeat may be, for example, between 2 and 50 units (i.e., repeats). In some instance, it may be

useful to have a new polypeptide with a number of repeat greater than 50. Examples of new polypeptides formed by the repetition of PCK3145 (SEQ ID NO: 5) are illustrated below (80 to 82). In some instance, SEQ ID NO: 5 units may be separated by a linker or an adaptor of variable length.

80) EWQTDNCETCTCYETEEWQTDNCETCTCYETE (SEQ ID NO: 90)

81) EWQTDNCETCTCYETEEWQTDNCETCTCYETEEWQTDNCETCTCYETE (SEQ ID NO: 91)

10 82) EWQTDNCETCTCYETEEWQTDNCETCTCYETEEWQTDNCETCTCYETEEWQTDNCE
TCTCYETE (SEQ ID NO: 92)

Heterologous fusion includes new polypeptides made by the fusion of polypeptides of the present invention with heterologous polypeptides. Such polypeptides may include but are not limited to bacterial polypeptides (e.g., betalactamase, glutathione-S-transferase, or an enzyme encoded by the E.coli trp locus), yeast protein, viral proteins, phage proteins, bovine serum albumin, chemotactic polypeptides, immunoglobulin constant region (or other immunoglobulin regions), albumin, or ferritin.

Other type of polypeptide modification includes amino acids sequence deletions (e.g., truncations). Those generally range from about 1 to 30 residues, more preferably about 1 to 10 residues and typically about 1 to 5 residues.

A host cell transformed or transfected with nucleic acids encoding the polypeptides of the present invention (i.e., vector containing the DNA sequence of the polypeptides of the present invention) or chimeric proteins formed with the polypeptides of the present invention are also encompassed by the invention. Any host cell, which produces a polypeptide analog, mutant, variant, fragment, or chimera having at least one of the biological properties of the present invention is encompassed by the present invention. For example, such host cell may include bacterial, yeast, plant, insect or mammalian cells. In addition, the polypeptides of the present invention may be produced in transgenic animals. Transformed or transfected host cells and transgenic animals may be obtained using materials and methods that are routinely available to one skilled in the art.

DEFINITIONS

General Molecular Biology

5 Unless otherwise indicated, the recombinant DNA techniques utilized in the present invention are standard procedures, known to those skilled in the art. Example of such techniques are explained in the literature in sources such as J. Perbal, A Practical Guide to Molecular Cloning, John Wiley and Sons (1984), J. Sambrook et al.,
10 Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press (1989), T.A. Brown (editor), Essential Molecular Biology: A Practical Approach, Volumes 1 and 2, IRL Press (1991), D.M. Glover and B.D. Hames (editors), DNA Cloning: A Practical Approach, Volumes 1-4, IRL Press (1995 and 1996), and F.M. Ausubel et al. (editors),
15 Current Protocols in Molecular Biology, Greene Pub. Associates and Wiley-Interscience (1988, including all updates until present) and are incorporated herein by reference.

"Polynucleotide" generally refers to any polyribonucleotide or
20 polydeoxyribonucleotide, which may be unmodified RNA or DNA, or modified RNA or DNA. "Polynucleotides" include, without limitation single- and double-stranded DNA, DNA that is a mixture of single- and double-stranded regions, single- and double-stranded RNA, and RNA that is a mixture of single- and double-stranded regions, hybrid
25 molecules comprising DNA and RNA that may be single-stranded or, more typically, double-stranded or a mixture of single- and double-stranded regions. In addition, "polynucleotide" refers to triple-stranded regions comprising RNA or DNA or both RNA and DNA. The term polynucleotide also includes DNAs or RNAs containing one or more
30 modified bases and DNAs or RNAs with backbones modified for stability or for other reasons. "Modified" bases include, for example, tritylated bases and unusual bases such as inosine. A variety of modifications has been made to DNA and RNA; thus "polynucleotide" embraces chemically, enzymatically or metabolically modified forms of
35 polynucleotides as typically found in nature, as well as the chemical forms of DNA and RNA characteristic of viruses and cells. "Polynucleotide" includes but is not limited to linear and end-closed molecules. "Polynucleotide" also embraces relatively short polynucleotides, often referred to as oligonucleotides.

40 "Polypeptides" refers to any peptide or protein comprising two or more amino acids joined to each other by peptide bonds or modified peptide bonds (i.e., peptide isosteres). "Polypeptide" refers to both short chains, commonly referred as peptides, oligopeptides or

oligomers, and to longer chains generally referred to as proteins. As described above, polypeptides may contain amino acids other than the 20 gene-encoded amino acids.

5 As used herein the term "polypeptide analog" relates to mutants, variants, chimeras, fusions, deletions, additions and any other type of modifications made relative to a given polypeptide.

10 As used herein, the term "homologous" sequence relates to nucleotide or amino acid sequence derived from the rHuPSP94 DNA sequence or polypeptide.

15 As used herein, the term "heterologous" sequence relates to DNA sequence or amino acid sequence of a heterologous polypeptide and includes sequence other than that of PSP94.

20 As used herein, the term "tumor" relates to solid or non-solid tumors, metastatic or non-metastatic tumors, tumors of different tissue origin including, but not limited to, tumors originating in the liver, lung, brain, lymph node, bone marrow, adrenal gland, breast, colon, pancreas, prostate, stomach, or reproductive tract (cervix, ovaries, endometrium etc.). The term "tumor" as used herein, refers also to all neoplastic cell growth and proliferation, whether malignant or benign, and all pre-cancerous and cancerous cells and
25 tissues.

30 As used herein, the term "polysaccharide" refers to a substance made of two or more saccharide unit and comprise, for example, chitosan, pectin, chondroitin sulfate, cyclodextrin, dextrans, guar gum, inulin, amylose, and locust bean gum.

35 As used herein, the term "vector" refers to an autonomously replicating DNA or RNA molecule into which foreign DNA or RNA fragments are inserted and then propagated in a host cell for either expression or amplification of the foreign DNA or RNA molecule. The term « vector » comprises and is not limited to a plasmid (e.g., linearized or not) that can be used to transfer DNA sequences from one organism to another.

40 As used herein, the term "time-release encapsulation means" refers to controlled or sustained release obtained when a pharmaceutical composition is formulated, for example, with polysaccharides, biocompatible polymers, other polymeric matrices, capsules, microcapsules, microparticles, bolus preparations, osmotic

pumps, diffusion devices, liposomes, lipospheres, dry powders, or transdermal delivery systems. Other controlled release compositions of the present invention include liquids that, upon administration to a mammal, form a solid or a gel in situ. Furthermore, the term "time-release encapsulation means" or "time-release means" comprises a class of biodegradable polymers useful in achieving controlled release of a drug, for example, polylactic acid, polyglycolic acid, copolymers of polylactic and polyglycolic acid, polyepsilon caprolactone, polyhydroxy butyric acid, polyorthoesters, polyacetals, polydihydropyrans, polycyanoacylates, and crosslinked or amphipathic block copolymers of hydrogels.

As used herein, "pharmaceutical composition" means therapeutically effective amounts of the agent together with pharmaceutically acceptable diluents, preservatives, solubilizers, emulsifiers, adjuvant and/or carriers. A "therapeutically effective amount" as used herein refers to that amount which provides a therapeutic effect for a given condition and administration regimen. Such compositions are liquids or lyophilized or otherwise dried formulations and include diluents of various buffer content (e.g., Tris-HCl., acetate, phosphate), pH and ionic strength, additives such as albumin or gelatin to prevent absorption to surfaces, detergents (e.g., Tween 20, Tween 80, Pluronic F68, bile acid salts). Solubilizing agents (e.g., glycerol, polyethylene glycerol), anti-oxidants (e.g., ascorbic acid, sodium metabisulfite), preservatives (e.g., thimerosal, benzyl alcohol, parabens), bulking substances or tonicity modifiers (e.g., lactose, mannitol), covalent attachment of polymers such as polyethylene glycol to the protein, complexation with metal ions, or incorporation of the material into or onto particulate preparations of polymeric compounds such as polylactic acid, polyglycolic acid, hydrogels, etc, or onto liposomes, microemulsions, micelles, unilamellar or multilamellar vesicles, erythrocyte ghosts, or spheroplasts. Such compositions will influence the physical state, solubility, stability, rate of in vivo release, and rate of in vivo clearance. Controlled or sustained release compositions include formulation in lipophilic depots (e.g., fatty acids, waxes, oils). Also comprehended by the invention are particulate compositions coated with polymers (e.g., poloxamers or poloxamines). Other embodiments of the compositions of the invention incorporate particulate forms protective coatings, protease inhibitors or permeation enhancers for various routes of administration, including parenteral, pulmonary, nasal and oral routes. In one embodiment the pharmaceutical composition is administered parenterally, paracancerally, transmucosally,

transdermally, intramuscularly, intravenously, intradermally, subcutaneously, intraperitoneally, intraventricularly, intracranially and intratumorally.

5 Further, as used herein "pharmaceutically acceptable carrier" or "pharmaceutical carrier" are known in the art and include, but are not limited to, 0.01-0.1 M and preferably 0.05 M phosphate buffer or 0.8 % saline. Additionally, such pharmaceutically acceptable carriers may be aqueous or non-aqueous solutions, suspensions, and emulsions.
10 Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium
15 chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers such as those based on Ringer's dextrose, and the like. Preservatives and other additives may also be present, such as, for example, antimicrobials,
20 antioxidants, collating agents, inert gases and the like.

Mutants, variants and analogs proteins

25 Mutant polypeptides will possess one or more mutations, which are deletions (e.g., truncations), insertions (e.g., additions), or substitutions of amino acid residues. Mutants can be either naturally occurring (that is to say, purified or isolated from a natural source) or synthetic (for example, by performing site-directed mutagenesis on the encoding DNA or made by other synthetic
30 methods such as chemical synthesis). It is thus apparent that the polypeptides of the invention can be either naturally occurring or recombinant (that is to say prepared from the recombinant DNA techniques).

35 A protein at least 50 % identical, as determined by methods known to those skilled in the art (for example, the methods described by Smith, T.F. and Waterman M.S. (1981) Ad. Appl.Math., 2:482-489, or Needleman, S.B. and Wunsch, C.D. (1970) J.Mol.Biol., 48: 443-453), to those polypeptides of the present invention are included in the
40 invention, as are proteins at least 70 % or 80 % and more preferably at least 90 % identical to the protein of the present invention. This will generally be over a region of at least 5, preferably at least 20 contiguous amino acids.

"Variant" as the term used herein, is a polynucleotide or polypeptide that differs from reference polynucleotide or polypeptide respectively, but retains essential properties. A typical variant of a polynucleotide differs in nucleotide sequence from another, reference polynucleotide. Changes in the nucleotide sequence of the variant may or may not alter the amino acid sequence of a polypeptide encoded by the reference polynucleotide. Nucleotide changes may result in amino acid substitutions, additions, deletions, fusion and truncations in the polypeptide encoded by the reference sequence, as discussed herein. A typical variant of a polypeptide differs in amino acid sequence from another, reference polypeptide. Generally, differences are limited so that the sequence of the reference polypeptide and the variant are closely similar overall and, in many regions, identical. A variant and reference polypeptide may differ in amino acid by one or more substitutions, additions, deletions, or any combination therefore. A substituted or inserted amino acid residue may or may not be one encoded by the genetic code. A variant polynucleotide or polypeptide may be a naturally occurring such as an allelic variant, or it may be a variant that is not known to occur naturally. Non-naturally occurring variants of polynucleotides and polypeptides may be made by mutagenesis techniques or by direct synthesis.

Amino acid sequence variants may be prepared by introducing appropriate nucleotide changes into DNA, or by in vitro synthesis of the desired polypeptide. Such variant include, for example, deletions, insertions, or substitutions of residues within the amino acid sequence. A combination of deletion, insertion and substitution can be made to arrive at the final construct, provided that the final protein product possesses the desired characteristics. The amino acid changes also may alter posttranslational processes such as changing the number or position of the glycosylation sites, altering the membrane anchoring characteristics, altering the intra-cellular location by inserting, deleting or otherwise affecting the transmembrane sequence of the native protein, or modifying its susceptibility to proteolytic cleavage.

It is to be understood herein, that if a "range" or "group" of substances (e.g. amino acids), substituents" or the like is mentioned or if other types of a particular characteristic (e.g. temperature, pressure, chemical structure, time, etc.) is mentioned, the present invention relates to and explicitly incorporates herein each and every specific member and combination of sub-ranges or sub-groups therein whatsoever. Thus, any specified range or group is to

be understood as a shorthand way of referring to each and every member of a range or group individually as well as each and every possible sub-ranges or sub-groups encompassed therein; and similarly with respect to any sub-ranges or sub-groups therein. Thus, for
5 example,

- with respect to a pressure greater than atmospheric, this is to be understood as specifically incorporating herein each and every individual pressure state, as well as sub-range,
10 above atmospheric, such as for example 2 psig, 5 psig, 20 psig, 35.5 psig, 5 to 8 psig, 5 to 35, psig 10 to 25 psig, 20 to 40 psig, 35 to 50 psig, 2 to 100 psig, etc.;
- with respect to a temperature greater than 100° C, this is to be understood as specifically incorporating herein each and every individual temperature state, as well as sub-
15 range, above 100° C, such as for example 101° C, 105° C and up, 110° C and up, 115° C and up, 110 to 135° C, 115° c to 135° C, 102° C to 150° C, up to 210° C, etc.;
- with respect to a temperature lower than 100° C, this is to be understood as specifically incorporating herein each and every individual temperature state, as well as sub-range,
20 below 100° C, such as for example 15° C and up, 15° C to 40° C, 65° C to 95° C, 95° C and lower, etc.;
- with respect to residence or reaction time, a time of 1 minute or more is to be understood as specifically incorporating herein each and every individual time, as well
30 as sub-range, above 1 minute, such as for example 1 minute, 3 to 15 minutes, 1 minute to 20 hours, 1 to 3 hours, 16 hours, 3 hours to 20 hours etc.;
- with respect to polypeptides, a polypeptide analog
35 consisting of at least two contiguous amino acids of a particular sequence is to be understood as specifically incorporating each and every individual possibility, such as for example, a polypeptide analog consisting of amino acid 1 and 2, a polypeptide analog consisting of amino acids 2 and 3, a polypeptide analog consisting of amino acids 3 and 4, a
40 polypeptide analog consisting of amino acids 6 and 7, a polypeptide analog consisting of amino acids 9 and 10, a polypeptide analog consisting of amino acids 36 and 37, a

polypeptide analog consisting of amino acids 93 and 94, etc.

- 5 - with respect to polypeptides, a polypeptide analog consisting of at least five contiguous amino acids of a particular sequence is to be understood as specifically incorporating each and every individual possibility, such as for example, a polypeptide analog consisting of amino acids 1 to 5, a polypeptide analog consisting of amino acids 2 to 6, a polypeptide analog consisting of amino acids 3 to 7, a
10 polypeptide analog consisting of amino acids 6 to 10, a polypeptide analog consisting of amino acids 9 to 13, a polypeptide analog consisting of amino acids 36 to 40, a polypeptide analog consisting of amino acids 90 to 94, etc.

- 15 - with respect to polypeptides, a polypeptide analog comprising a particular sequence and having an addition of at least one amino acid to its amino-terminus is to be understood as specifically incorporating each and every
20 individual possibility, such as for example, a polypeptide analog having an addition of one amino acid to its amino-terminus, a polypeptide analog having an addition of two amino acid to its amino-terminus, a polypeptide analog having an addition of three amino acid to its amino-terminus, a polypeptide analog having an addition of ten
25 amino acid to its amino-terminus, a polypeptide analog having an addition of eighteen amino acid to its amino-terminus, a polypeptide analog having an addition of forty amino acid to its amino-terminus, a polypeptide analog having an addition of two hundred amino acid to its amino-terminus, etc.
30

- 35 - with respect to polypeptides, a polypeptide analog comprising a particular sequence and having an addition of at least one amino acid to its carboxy-terminus is to be understood as specifically incorporating each and every individual possibility, such as for example, a polypeptide
40 analog having an addition of one amino acid to its carboxy-terminus, a polypeptide analog having an addition of two amino acid to its carboxy-terminus, a polypeptide analog having an addition of five amino acid to its carboxy-terminus, a polypeptide analog having an addition of twenty amino acid to its carboxy-terminus, a polypeptide analog having an addition of fifty-three amino acid to its carboxy-terminus, a polypeptide analog having an addition of three

hundred amino acid to its carboxy-terminus, etc.

- with respect to polypeptides, a polypeptide analog comprising two to fifty units of a particular sequence is to be understood as specifically incorporating each and every individual possibility, such as for example, a polypeptide analog comprising two units of that particular sequence, a polypeptide analog comprising three units of that particular sequence, a polypeptide analog comprising six units of that particular sequence, a polypeptide analog comprising thirteen units of that particular sequence, a polypeptide analog comprising thirty-five units of that particular sequence, a polypeptide analog comprising fifty units of that particular sequence, etc.

- with respect to polypeptides, a polypeptide analog comprising two to ten units of a particular sequence is to be understood as specifically incorporating each and every individual possibility, such as for example, a polypeptide analog comprising two units of that particular sequence, a polypeptide analog comprising three units of that particular sequence, a polypeptide analog comprising four units of that particular sequence, a polypeptide analog comprising five units of that particular sequence, a polypeptide analog comprising six units of that particular sequence, a polypeptide analog comprising seven units of that particular sequence, a polypeptide analog comprising eight units of that particular sequence, a polypeptide analog comprising nine units of that particular sequence, and a polypeptide analog comprising ten units of that particular sequence.

- with respect to polypeptides, a polypeptide analog consisting of a sequence of from two to fourteen amino acid units wherein the amino acid units are selected from the group of amino acid units of SEQ ID NO: 5 consisting of glutamic acid (Glu), tryptophan (Trp), glutamine (Gln), threonine (Thr), aspartic acid (Asp), asparagine (Asn), cysteine (Cys), or tyrosine (Tyr), is to be understood as specifically incorporating each and every individual possibility, such as for example, a polypeptide analog of two amino acid units wherein the amino acids are sequentially; Glu and Trp, a polypeptide analog of two amino acid units wherein the amino acids are sequentially; Trp and Glu, a polypeptide analog of three amino acid units wherein

the amino acids are sequentially; Trp, Glu, Trp, a polypeptide analog of three amino acid units wherein the amino acids are sequentially; Trp, Trp, Trp, a polypeptide analog of three amino acid units wherein the amino acids are sequentially; Glu, Glu, Trp, a polypeptide analog of three amino acid units wherein the amino acids are, independently of the order; Tyr, Asp, Glu, a polypeptide analog of three amino acid units wherein the amino acids are, independently of the order; Thr, Asp, Asn, a polypeptide analog of three amino acid units wherein the amino acids are, independently of the order; Thr, Thr, Asn, a polypeptide analog of four amino acid units wherein the amino acids are, independently of the order; Glu, Gln, Cys, Asn, a polypeptide analog of four amino acid units wherein the amino acids are, independently of the order; Gln, Gln Cys, Trp, a polypeptide analog of four amino acid units wherein the amino acids are, Cys, Cys, Cys, Cys, a polypeptide analog of fourteen amino acid units wherein the amino acids are, independently of the order; Asn, Asp, Glu, Gln, Trp, Cys, Tyr, Thr, Thr, Asp, Asn, Gln, Thr, Cys, a polypeptide analog of fourteen amino acid units wherein the amino acids are, independently of the order; Asp, Asp, Asp, Asp, Trp, Cys, Cys, Trp, Thr, Thr, Thr, Thr, Thr, Cys, a polypeptide analog of fourteen amino acid units wherein the amino acids are, independently of the order; Tyr, Tyr, Tyr, Tyr, Tyr, Tyr, Tyr, Tyr, Tyr, Tyr, Tyr, Tyr, Tyr, Tyr, etc.

- with respect to polypeptides, a polypeptide analog having at least 90 % of its amino acid sequence identical to a particular amino acid sequence is to be understood as specifically incorporating each and every individual possibility (excluding 100 %), such as for example, a polypeptide analog having 90 % of its amino acid sequence identical to that particular amino acid sequence, a polypeptide analog having 91 % of its amino acid sequence identical to that particular amino acid sequence, a polypeptide analog having 93 % of its amino acid sequence identical to that particular amino acid sequence, a polypeptide analog having 97 % of its amino acid sequence identical to that particular amino acid sequence, a polypeptide analog having 99 % of its amino acid sequence identical to that particular amino acid sequence, etc.

with respect to polypeptides, a polypeptide analog having at

- least 70 % of its amino acid sequence identical to a particular amino acid sequence is to be understood as specifically incorporating each and every individual possibility (excluding 100 %), such as for example, a
- 5 polypeptide analog having 70 % of its amino acid sequence identical to that particular amino acid sequence, a polypeptide analog having 71 % of its amino acid sequence identical to that particular amino acid sequence, a
- 10 polypeptide analog having 73 % of its amino acid sequence identical to that particular amino acid sequence, a polypeptide analog having 88 % of its amino acid sequence identical to that particular amino acid sequence, a
- 15 polypeptide analog having 97 % of its amino acid sequence identical to that particular amino acid sequence, a polypeptide analog having 99 % of its amino acid sequence identical to that particular amino acid sequence, etc.
- with respect to polypeptides, a polypeptide analog having at least 50 % of its amino acid sequence identical to a
- 20 particular amino acid sequence is to be understood as specifically incorporating each and every individual possibility (excluding 100 %), such as for example, a polypeptide analog having 50 % of its amino acid sequence identical to that particular amino acid sequence, a
- 25 polypeptide analog having 51 % of its amino acid sequence identical to that particular amino acid sequence, a polypeptide analog having 54 % of its amino acid sequence identical to that particular amino acid sequence, a
- 30 polypeptide analog having 66 % of its amino acid sequence identical to that particular amino acid sequence, a polypeptide analog having 70 % of its amino acid sequence identical to that particular amino acid sequence, a
- 35 polypeptide analog having 79 % of its amino acid sequence identical to that particular amino acid sequence, a polypeptide analog having 82 % of its amino acid sequence identical to that particular amino acid sequence, a
- 40 polypeptide analog having 99 % of its amino acid sequence identical to that particular amino acid sequence, etc.
- and similarly with respect to other parameters such as low pressures, concentrations, elements, etc...

It is also to be understood herein that "g" or "gm" is a reference to the gram weight unit; that "C" is a reference to the

Celsius temperature unit; and "psig" is a reference to "pounds per square inch gauge".

5 BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts mass spectrometry analysis of polypeptide 7-21 (SEQ ID NO: 4).

10 Figure 2 depicts mass spectrometry analysis of polypeptide PCK3145 (SEQ ID NO: 5).

Figure 3 depicts mass spectrometry analysis of polypeptide 76-94 (SEQ ID NO: 6).

15 Figure 4a is a graph depicting the in-vitro inhibitory activity of the decapeptide of SEQ ID NO: 3 on PC-3 cells after 9 days of culture.

20 Figure 4b is a graph depicting the in-vitro inhibitory activity of the native PSP94 (nPSP94) on PC-3 cells after 9 days of culture.

Figure 5a is a graph depicting the in-vitro inhibitory activity of the decapeptide of SEQ ID NO: 3 on PC-3 cells after 21 days of
25 culture.

Figure 5b is a graph depicting the in-vitro inhibitory activity of the native PSP94 (nPSP94) on PC-3 cells after 21 days of culture.

30 Figure 6a is a graph depicting the in-vitro inhibitory activity of the decapeptide of SEQ ID NO: 3 on PC-3 cells after 10 days of culture.

Figure 6b is a graph depicting the in-vitro inhibitory activity of
35 the native PSP94 (nPSP94) on PC-3 cells after 10 days of culture.

Figure 7 depicts a gel showing DNA fragmentation following treatment of PC-3 cells with polypeptide PCK3145 as set forth in SEQ ID NO: 5.

40 Figure 8 is a graph depicting the results of an apoptosis assay with an ELISA plus kit following polypeptide treatment of PC-3 cells for 72 hours with various concentration of polypeptide 7-21 (SEQ ID NO: 4), polypeptide PCK3145 (SEQ ID NO: 5), polypeptide 76-94 (SEQ ID NO: 6) or native PSP94 (SEQ ID NO: 1).

45

Figure 9 is a graph depicting in vitro fibroblast cell growth when exposed for 72 hours to various concentration of native PSP94 (nPSP94) (SEQ ID NO: 1) or various concentration of rHuPSP94 (SEQ ID NO: 2) or polypeptide 7-21 (SEQ ID NO: 4), polypeptide PCK3145 (SEQ ID NO: 5), or polypeptide 76-94 (SEQ ID NO: 6).

Figure 10 is a graph depicting the effect of polypeptide 7-21 (SEQ ID NO: 4), polypeptide PCK3145 (SEQ ID NO: 5), polypeptide 76-94 (SEQ ID NO: 6), and polypeptide 61-75 on the in vitro growth of PC-3 cells after 72 hours.

Figure 11 is a graph depicting the effect of polypeptide 22-36 and polypeptide PCK3145 (SEQ ID NO: 5) on in vitro growth of PC-3 cells after 72 hours.

Figure 12 is a graph depicting results of study no. MLL-1 on the anti-tumor efficacy validation of rHuPSP94 (rPSP94) (SEQ ID NO: 2) against Mat Ly Lu (MLL) tumor implanted in nude mice.

Figure 13 is a graph depicting results of study no. MLL-2 on the anti-tumor efficacy validation of rHuPSP94 (rPSP94) (SEQ ID NO: 2) against Mat Ly Lu (MLL) tumor implanted in nude mice.

Figure 14 is a graph depicting tumor volume (tumor growth reduction) in rHuPSP94-treated nude mice.

Figure 15 is a graph depicting tumor volume (tumor growth reduction) in decapeptide (SEQ ID NO: 3)-treated nude mice.

Figure 16 is a graph depicting tumor volume (tumor growth reduction) in control scrambled polypeptide (PB111)-treated mice.

Figure 17 is a graph depicting tumor volume (tumor growth reduction) in native-PSP94 (nPSP94)-treated mice.

Figure 18 is a graph depicting the in vitro inhibitory activity of PCK3145 (SEQ ID NO: 5) on PC-3 cells, after a 72 hours treatment, as measured by MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt) assay.

Figure 19 is a graph depicting the in vitro inhibitory activity of native PSP94 (SEQ ID NO: 1) and PCK3145 (SEQ ID NO: 5) (GMP grade) on PC-3 cells, after 48 hours of treatment, as measured by MTS assay.

Figure 20 is a graph depicting the in vitro inhibitory activity of PCK3145 (SEQ ID NO: 5) (GMP grade) on PC-3 cells (ATCC), after 72 hours of treatment, as measured by the MTS assay.

5

Figure 21 is a graph depicting the in vitro inhibitory activity of PCK3145 (SEQ ID NO: 5) (GMP grade) on PC-3 cells (ATCC), after a 48 or 72 hours treatment, as measured by the MTS assay.

10 Figure 22 is a graph depicting the in vitro inhibitory activity of decapeptide as set forth in SEQ ID NO: 3, polypeptide 7-21 as set forth in SEQ ID NO: 4, polypeptide PCK3145 as set forth in SEQ ID NO: 5, or polypeptide 76-94 as set forth in SEQ ID NO: 6 on PC-3 cells, measured by [³H]-Thymidine uptake assay.

15

Figure 23 is a graph depicting the in vitro inhibitory activity of decapeptide as set forth in SEQ ID NO: 3, polypeptide 7-21 as set forth in SEQ ID NO: 4, polypeptide PCK3145 as set forth in SEQ ID NO: 5, or polypeptide 76-94 as set forth in SEQ ID NO: 6 on PC-3 cells, measured by [³H]-Thymidine uptake assay.

20

Figure 24 is a graph depicting the in vitro inhibitory activity of native PSP94 (SEQ ID NO: 1) on PC-3 cells after 72 hours treatment, measured by [³H]-Thymidine uptake assay.

25

Figure 25 depicts a gel showing DNA fragmentation following treatment of PC-3 cells with PCK3145 (SEQ ID NO: 5) or doxorubicin.

Figure 26 is a graph depicting the in vivo inhibitory activity of PCK3145 (SEQ ID NO: 5) (0.1 µg/kg/day and 10 µg/kg/day) against human PC-3 tumor xenografted in nude mice.

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Figure 27 is a graph depicting the in vivo inhibitory activity of PCK3145 (SEQ ID NO: 5) (10 µg/kg/day to 1000 µg/kg/day, administered either via the intra-venous or intra-peritoneal route) against human PC-3 tumor xenografted in nude mice.

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Figure 28 is a graph depicting the in vivo inhibitory activity of polypeptide 7-21 (SEQ ID NO: 4), PCK3145 (SEQ ID NO: 5) or polypeptide 76-94 (SEQ ID NO: 6), given at doses of 1 µg/kg/day or 10 µg/kg/day, in Copenhagen rats implanted with Dunning Mat Ly Lu tumors.

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Figure 29 is a graph depicting the in vivo inhibitory activity of PCK3145 (SEQ ID NO: 5) or the scrambled polypeptide given at doses of 10 µg/kg/day or 100 µg/kg/day, in Copenhagen rats implanted with Dunning Mat Ly Lu tumors.

Figure 30 is a graph depicting tumor weight at day 18 following PCK3145 (SEQ ID NO: 5) or scrambled polypeptide treatment (10 µg/kg/day or 100 µg/kg/day), in Copenhagen rats implanted with Dunning Mat Ly Lu tumors.

Figure 31 is a graph depicting the efficacy of PCK3145 and taxotere (i.e. docetaxel) combination treatment in Nude mice implanted with PC-3 tumor cells in tumor growth retardation.

DETAILED DESCRIPTION OF THE INVENTION

The recombinant human rHuPSP94 expressed in yeast is non-glycosylated and has 10 cystein residues. The molecular weight of rHuPSP94 was determined to be 11.5 kDa, compared to 10.7 kDa for its native counterpart.

Various experimental studies have been carried out in order to determine the efficacy of rHuPSP94 (SEQ ID NO: 2) relative to the native PSP94 secreted by the diseased prostate as tumor suppressive agent. Studies have also been carried out to determine the efficacy of the decapeptide as set forth in SEQ ID NO: 3, the polypeptide as set forth in SEQ ID NO: 4 (polypeptide 7-21), the polypeptide as set forth in SEQ ID NO: 5 (PCK3145), and the polypeptide as set forth in SEQ ID NO: 6 (polypeptide 76-94), as tumor suppressive agents. The tumor suppression activity of the polypeptides of the present invention has been monitored by their ability to reduce or inhibit the growth of prostatic adenocarcinoma both in-vivo and in-vitro. Those results are summarized below.

Studies were carried out using PC-3 human prostate adenocarcinoma line, which can be maintained both in vivo as a xenograft in nude mice and in vitro as a cell line. In addition, a rat Dunning Mat LyLu prostate tumor, which is a pre-eminent animal model for the study of CaP, was also used. The Dunning tumor is a fast growing, poorly differentiated, transplantable tumor, which can be maintained both in-vivo in the Copenhagen rat and in-vitro as a cell line.

The following examples are offered by way of illustration and not by way of limitation.

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EXAMPLE 1

PREPARATION OF rHuPSP94 (SEQ ID NO: 2) and polypeptides (SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5 and SEQ ID NO: 6)

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Recombinant HuPSP94 was cloned and expressed in *Pichia pastoris*, and then purified and characterized as follows.

Materials

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DEAE-cellulose (DE52) was purchased from Whatman (Fairfield, New-Jersey). Dialysis membranes and the electro chemiluminescence (ECL) detection kit were purchased from Biolynx Canada (Pierce Inc.). Broad-range molecular weight markers and Econo-pack columns fitted with flow adapters were purchased from Bio-Rad Labs Ltd (California). Pellicon device was purchased from Millipore (Massachusetts). Tris-HCl was obtained from ICN. MES ((2-[N-Morpholino]ethanesulfonic acid) hydrate) was obtained from Sigma. Swine anti-rabbit IgG alkaline-phosphatase conjugates was purchased from DAKO (Denmark). *Pichia Pastoris* expression Kit version G was from Invitrogen (Carlsbad, California). Non-Radioactive High Prime DIG labeling kit® was purchased from Boehringer Mannheim (Indianapolis, Indiana). The MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) assays were performed using Cell Titer Aqueous Non radioactive cell proliferation assay kit from Promega (Madison, Wisconsin). MRX microtiter plate reader was from Dynex technologies (Chantilly, Virginia). Rabbit polyclonal antiserum against PSP94 was a gift from the late Dr. A. Sheth. All primers were synthesized by Procyon Biopharma Inc. London, Ontario, Canada.

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Cell line and cell culture

P. pastoris host strain GS115 (*his4*) and all *Pichia* related products were obtained from Invitrogen. PC-3 (ATCC-# CRL 1435) cell line was obtained from the American Type Cell Culture (ATCC) and maintained in OPTI MEM (minimum essential media) with 10 % fetal bovine serum (FBS). All cell culture products were obtained from GIBCO BRL.

Cloning

TA cloning vector (pCR TM 2.1) containing human PSP94 cDNA including a 20 amino acid leader sequence described previously (Baijal-Gupta, M., et. al., J. Endocrinol., 165:425-433, 2000) was used to amplify human PSP94 without its leader sequence using appropriate primers. The primers for the polymerase chain reaction (PCR) were designed to contain an EcoRI restriction sites at either end. The 5' primer used was 5'-GGG AAG AAT TCT CAT GCT ATT TCA TA-3' (SEQ ID NO: 7) and the 3' primer, 5'-TGG ATA TCT GCA GAA TTC GGC-3' (SEQ ID NO: 8). The +1 start site for PSP94 (at a Serine residue) has been underlined in the 5' primer described above.

The PCR included 1 cycle of 12 minutes at 94 °C, followed by 25 cycles of 1 minute at 94 °C, 1 minute at 55 °C, 1 minute at 72 °C and a final step of 1 cycle of 10 minutes at 72 °C. PCR amplification of the product was performed using BM ExpandTM High Fidelity PCR System. The product was run on a 1.5 % agarose gel and the appropriate PCR product was isolated using Pharmacia Sephaglass Kit (Bandprep). Subcloning of the PSP94 insert was performed in pPIC9 vector (Invitrogen). The EcoRI enzyme was used for the restriction digestion of both the plasmid and the PCR products (thus removing PSP94 signal sequence) followed by ligation and transformation, using DH5 α cells. The isolated clones were selected for by ampicillin resistance and inserts were identified by restriction mappings. The constructs were sequenced (Robart's sequencing service, London, Ontario) to identify PSP94 insert with a correct sequence as well as proper orientation and reading frame.

Screening for Clones Expressing rHuPSP94

For *Pichia pastoris* transformation, the spheroplast method was used according to manufacturer's instructions (Invitrogen) using GS115 and KM71 yeast strains. Plasmid pPIC9 with or without the PSP94 insert were linearized using SalI restriction enzyme. Transformed colonies were screened and selected for their ability to produce their own histidine, hence survived on media without histidine. All GS115 transformants scored as Mut⁺, whereas all KM71 colonies, which did not grow well in the liquid culture, scored as Mut⁻. Hence a number of GS115 clones were screened for production of the highest levels of rHuPSP94 expression.

About a hundred clones were selected and grown into 2 ml of culture media until an optical density at 600 nm (OD₆₀₀) of approximately 6 was reached. Total DNA was isolated for rapid dot

blot analysis in order to detect multiple integrations by Southern blot that would possibly correspond to high rHuPSP94 expressing clones. Two hundred microliters of each culture specimens were denatured and blotted (in duplicate) to a positively charged nylon
5 membrane, placed in a dot blot apparatus. The membrane was subsequently air-dried. The membrane was soaked between two sheets of Whatman 3MM paper for 15 minutes in a solution containing 50mM ethylenediaminetetraacetic acid (EDTA), 2.5 % beta-mercaptoethanol (BME), pH 9, followed by an incubation of 24 hours at 37 °C with 1
10 mg/ml Zymolyase 100T, 5 minutes in 0.1 N NaOH, 1.5 M NaCl, 0.015 M sodium citrate pH 7 and two 5 minutes incubation in 2x saline-sodium citrate (SSC). Finally the membrane was baked at 80 °C for 45 minutes and exposed to ultraviolet light (UV) for 15 minutes. Human PSP94 cDNA probe was labeled with the non-radioactive High Prime DIG
15 labeling kit® (Boehringer Mannheim) and was used for hybridization. Hybridization with digoxigenin labeled cDNA probe (25ng/μl) was done for 2 days at 42 °C in Sodium dodecyl sulfate (SDS) buffer (SDS 7 % (w/v); formamide 50 % (v/v); 5 X SSC; 50 mM sodium phosphate, pH 7.0; N-lauroyl-sarcosine 0.1 % (w/v)) and blocking reagent, CSPD® 2 %
20 (w/v) (Boehringer Mannheim) was used as the chemiluminescence substrate. All digoxigenin (DIG) labeling procedures were performed according to the manufacturer's instruction. Detection was performed using the Hyper film-ECL product (Amersham Life Science Inc. Arlington Hts, Illinois).
25 The clone with the highest signal intensity was used for all flasks shaken cultures.

Optimization of the Expression of the Protein in Flask Shaken Cultures

30 A clone containing the PSP94 construct was selected for high expression of the protein. Colony was grown in 25ml of basal minimum growth media (BMG) until an OD600 between 2 and 6 was obtained. This clone was further amplified in Baffled Erlenmeyer flasks in a volume of 1 liter of BMG media until the OD600 reached approximately between
35 2.0 to 6.0. The culture was centrifuged for 15 minutes at 2500 X g and the pellet was collected. The induction phase (i.e., induction of expression of rHuPSP94) was carried out by inoculating the cell pellet in basal minimum media (BMM). Growth was performed in Baffled flasks for 6 days, as recommended by Invitrogen. The volume of BMM
40 added varied according to the size of the pellet collected. Five milliliters of 100 % methanol were added for each liter of culture. This was performed each day, around the same time, to a final

concentration of 0.1 % of methanol. A plasmid without the PSP94 insert served as a negative control.

To determine the optimum time for harvesting rHuPSP94 secreted in the cell culture media, aliquots were taken every 24 hours for 6 days, starting from the first day of induction. Levels of rHuPSP94 protein expression were determined by measuring OD600 and by performing a 15 % SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) stained with Coomassie Brilliant blue or by Western blot analysis using polyclonal antibody against PSP94.

Sample Preparation

Culture supernatant of clone showing the highest rHuPSP94 expression, post-induction (e.g., after 96 hours), was centrifuged at 2500 X g for 20 minutes. The supernatant was filtered through a 0.8 µm filter and concentrated approximately 10-fold using a Pellicon unit (Millipore). The filtered supernatant was dialyzed against 0.05 mM Tris-HCl buffer, pH 8.0, using a 3500 molecular weight cut-off membrane. An aliquot of the dialyzed supernatant was analyzed by SDS-PAGE and Western blot analysis and the rest was submitted to further purification.

Culture Conditions for Fermentation

Fermentation was carried out at the Institute for Biological Sciences, National Research Council (NRC) (Ottawa, Ontario Canada), following manufacturer's instruction (Invitrogen). For example, a fermentation procedure was initiated by inoculating 7.5 liter of media with 625 ml of a starting culture. The growth phase was carried out for approximately 2 days in BMG media until the OD600 reached approximately 0.5. The induction phase was initiated by the addition of methanol (100 %), according to the manufacturer's instructions (Invitrogen). The culture was harvested after 95 hours (i.e., after induction with methanol for 67 hours). The final volume of the culture was approximately 13.5 liters.

Sample Preparation from Fermentation Culture

The large cell mass was removed by centrifugation. The cell free media collected (9 liters) was further clarified using a 0.2 µm filtration unit (Pellicon). The remaining 8.5 liters containing secreted rHuPSP94 was tested for protein expression and stored at -20 °C for further isolation and purification of the protein.

Protein Estimation

The amount of rHuPSP94 protein secreted in the culture supernatant from the flask shaken and the fermentation process was obtained based on estimates of band intensities of samples compared to band intensities of a standard curve obtained by loading known quantities of pure lyophilized PSP94 on a SDS-PAGE. The initial estimate for rHuPSP94 at each step of purification was determined by OD at 280 nm. Quantification of total protein content at the final steps of purification was done by the BCA (bicinchoninic acid) method, using bovine serum albumin (BSA) as standard.

Lyophilization

Samples of purified rHuPSP94 were dialyzed against deionized water using a 3000 molecular weight cut-off membrane and were lyophilized.

SDS-PAGE

SDS-PAGE was performed using acrylamide at a final concentration of 15 % for the separating portion of the gel and acrylamide at a final concentration of 5 % for the stacking portion of the gel. The gel contained 0.1 % SDS and was performed under reducing conditions. Broad-range molecular weight markers were used for the estimation of molecular weight of the protein. Proteins were stained with Coomassie Brilliant Blue R-250.

Western Blotting

For immunoblotting, Mini Trans-Blot Electrophoretic Transfer Cell (Bio Rad) was used with Hi bond-C super membrane (Amersham) and 85mm blotting papers. Protein samples (0.4 µg) were loaded and separated on SDS-PAGE, as described earlier. Proteins were transferred to the membrane for 2 hours at 4 °C, using transfer buffer (25 mM Tris, 192 mM Glycine, pH 8.3 and 20 % methanol) and a transfer unit set at 200 milliamperes (mAmp). Membranes were blocked overnight by incubation in 2 % (w/v) non-fat dry milk (skim milk) dissolved in tris buffer saline (TBS: 500 mM NaCl, 20 mM Tris-HCl, pH 7.5) at room temp (RT). Membranes were washed three times with TBS containing 0.02 % (v/v) Tween-20 (this buffer is named TTBS). Membranes were subsequently incubated for 2 hours at RT with anti-PSP94 antibody (1:2000 dilution) diluted in TTBS containing 2 % skim milk. Membranes were washed twice with TTBS (5 minutes each washing), and incubated at RT with a secondary antibody (i.e., swine anti-rabbit antibody HRP conjugated) (1:5000 dilution) diluted in TTBS. Membranes were washed twice with 0.02 % TTBS (5 minutes each washing). Blots were developed using the ECL detection system,

according to manufacturer's instructions, using the Super Signal Substrate, and exposed to a Hyperfilm ECL from Amersham LS for 5 to 20 seconds. Pre-stained molecular weight markers were used for molecular weight estimation.

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Purification of rHuPSP94 using DE52 Column Chromatography

Following removal of *P. pastoris* cells from the fermentation culture, supernatant was concentrated approximately ten fold, dialyzed and subjected to anion exchange chromatography. A DE52 column having a bed volume of approximately 40 ml (2.5 cm internal diameter (id) X 8 cm height(h)) was equilibrated with 0.05 M Tris-HCl, pH 8.0 (equilibrating buffer). The sample (25 ml) containing 15 to 20 mg of rHuPSP94 protein was applied to the DE52 column at a flow rate of 1 ml/minute.

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Impurities were removed from the column by washing it with 40 to 50 ml of the equilibrating buffer, and monitoring the absorbance at 280 nm. This step was followed by the addition of 100 to 150 ml of 0.05 M Tris-HCl, pH 6.5 to the column until the pH of the wash reached approximately 6.5. The column was further washed with 100 to 150 ml of 0.05 M MES-acetate buffer, pH 6.5, until the absorbance at 280nm approached zero. Finally rHuPSP94 was eluted from the column with 0.05 M MES-acetate buffer, pH 5.0. Peak fractions were characterized by absorbance at 280 nm, followed by SDS-PAGE and Western blot analysis as described above. Fractions with high absorbance at 280 nm values (0.5 to 1.8) were pooled and dialyzed against water or PBS for storage at -20 °C and/or lyophilization.

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Amino acid Composition

Amino acid analysis of the DE52 purified flask shaken culture and fermentation cultures was carried out. The Perkin Elmer Biosystems Derivatizer-Analysis system was used with Spheri-5 PTC C-18 5µ column and UV detection at OD254.

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Mass Spectral analysis

PSP94 derived polypeptides were synthesized, were found to be in accordance with the required specifications and were analyzed by Mass Spectral Analysis. Mass spectrometry analysis of polypeptide 7-21 (SEQ ID NO: 4), PCK3145 (SEQ ID NO: 5) and polypeptide 76-94 (SEQ ID NO: 6) are represented in figures 1, 2 and 3 respectively.

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Polypeptide samples were analyzed using the PerSeptive Biosystems (Framingham, MA), with Voyager-DE MALDI-TOF mass

spectrometer using 337 nm light from a nitrogen laser. About 12 to 50 scans were averaged for each analysis.

Purified samples from the flask shaken culture and fermentation culture were analyzed using the PerSeptive Biosystems (Framingham, MA), with Voyager-DE MALDI-TOF mass spectrometer using 337 nm light from a nitrogen laser. About 50 scans were averaged for each analysis. A sample from the native PSP94 was also analyzed under similar conditions for comparison.

EXAMPLE 2

IN-VITRO EFFECT OF rHuPSP94 ON PC-3 CELLS (MTS ASSAY)

The biological activity of the rHuPSP94 was determined by its growth inhibitory effect on human prostate cancer cells PC-3. Cell proliferation was monitored on PC-3 cells using the MTS/PMS (phenazine methosulfate) kit (Promega), which primarily measures mitochondrial activity of live cells. The basic principle of this method involves the fact that the mitochondrial enzymes of the live cells metabolize the MTS/PMS dyes forming a brown colored precipitate which can be measured as optical density (OD) by absorption at 490 nm in a spectrophotometer. Therefore, the OD values are proportional to the number of living cells. In addition, monitoring of cell morphology was also performed. Cell morphology would be indicative of their health status. For example, viable cells would appear adherent and spread out whereas dead cells would be in suspension in the media and would appear granular and round.

Results of in vitro effect of rHuPSP94 on PC-3 cells measured by MTS assay are summarized in table 2, below. PC-3 cells (ATCC, Lot AT06) used in these experiments were at a passage number lower or equal to 70 ($n \geq 70$). Cells were seeded in Costar 96 well cell culture flat bottom plates in RPMI supplemented media containing 50 $\mu\text{g/ml}$ of bovine serum albumin (BSA) and 0.1 μM FeSO_4 . Peptide was diluted in the same media. Cells were continuously exposed to the polypeptides of the present invention for 72 hours without changing media. Native PSP94 or rHuPSP94 concentrated two fold were directly added to wells and diluted to 1X in order to minimize cell manipulation and avoid detachment.

The evaluation of growth inhibitory effect of rHuPSP94 on PC-3 cells indicated a substantial reduction in cell numbers (i.e.,

viability) ranging from 37 % to 57 % reduction at concentrations of 80 and 120 µg/ml of rHuPSP94 respectively. This effect was observed in 3 out of 4 experiments (Table 2). Results of trypan blue exclusion test demonstrated a cell viability of 62 % at 80 µg/ml.

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TABLE 2

Experiment no.	Sample	% Viability (control = 100%) (µg/ml)			
		40	60	80	120
1	rHuPSP94	72	78	58	43
2	rHuPSP94	63	63	63	68
3	rHuPSP94	95	85	78	ND
4	rHuPSP94	100	52	62	60
5	rHuPSP94	100	98	90	52
Sample	% Viability (control = 100%) (µg/ml)				
	5	10	20	40	80
rHuPSP94	98	84	78	70	55
rHuPSP94	92	95	80	71	59
rHuPSP94	89	69	79	68	65

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EXAMPLE 3

IN-VITRO EFFECT OF rHuPSP94 ON PC-3 CELLS
([³H]-THYMIDINE UPTAKE ASSAY)

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The in vitro growth inhibition effect of rHuPSP94 was assessed using [³H]-Thymidine uptake assay. [³H]-Thymidine uptake assay involves [³H]-Thymidine incorporation into cellular DNA of actively proliferating cells. It measures the proliferative index of the cells versus the MTS assay, which quantifies the number of lived cells following treatment. Cells were seeded in Costar 96 well cell culture flat bottom plates in RPMI supplemented media containing 50 µg/ml of bovine serum albumin (BSA) and 0.1 µM FeSO₄. PC-3 cells were exposed to various concentrations of rHuPSP94 for 72 hours and during the final 16 hours of incubation cells were pulsed with 1 µCi of [³H]-Thymidine. The radioactivity in each well of the plate is counted by a beta-counter and is expressed as total counts per minutes (cpm). Results of in vitro effect of rHuPSP94 on PC-3 cells using the [³H]-Thymidine uptake assay are summarized in Table 3 and are expressed as percentage of radioactivity measured for treated-cells relative to

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the radioactivity measured for non-treated cells (for which [³H]-thymidine uptake value was set at 100 %).

Results indicated a 65 % reduction in the percentage of cells incorporating [³H]-thymidine following treatment with rHuPSP94 at a concentration of 80 µg/ml for 72hrs, compared to the non-treated control. Results of a 65 % reduction in [³H]-thymidine uptake may also be an indication of a 65 % reduction in cell proliferation.

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Comparison was performed between [³H]-Thymidine uptake assay and the MTS assay, in order to evaluate their relative sensitivity. An additional plate was set aside for MTS assay and treated in parallel with the same lot (i.e., batch) of rHuPSP94 as the one used for the [³H]-thymidine uptake assay. Result obtained for the MTS assay demonstrated a 35 % reduction in cell viability (65 % cells remaining viable) following treatment with rHuPSP94 at a concentration of 80 µg/ml, indicating that the [³H]-Thymidine uptake assay, which was able to measure a 65 % reduction in cell proliferation, may be more sensitive than the MTS assay.

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TABLE 3

Experiment no.	Sample	³ [H]-Thymidine Uptake (% of control) (µg/ml)				
		5	10	20	40	80
1	rHuPSP94	94	101	98	79	35
1	native PSP94	97	98	100	98	77

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EXAMPLE 4

IN-VITRO EFFECT OF DECAPEPTIDE AND OTHER POLYPEPTIDE ON PC-3 CELLS

The synthetic decapeptide (SEQ ID NO: 3) has been shown herein to mimic the biological activity of native PSP94 (nPSP94) (SEQ ID NO: 1) and therefore its effect on the PC-3 cells was studied in clonogenicity assay (colony formation). Cells were seeded in Costar 96 well cell culture flat bottom plates in RPMI supplemented media containing 50 µg/ml of bovine serum albumin (BSA) and 0.1 µM FeSO₄. Clonogenicity was evaluated for PC-3 cells grown in the presence of various concentration of the decapeptide after 9 days of culture (Figure 4a). A parallel experiment was performed with various

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concentration of nPSP94 using the same experimental conditions (Figure 4b). Other experiments evaluating clonogenicity was performed with the decapeptide (Figure 5a) or nPSP94 (Figure 5b) after 21 days of culture as well as after 10 days of culture (Figure 6a: Decapeptide and Figure 6b: nPSP94).

Referring to Figures 4 to 6, the decapeptide (SEQ ID NO: 3) had a similar inhibitory action as nPSP94 (SEQ ID NO: 1) on in-vitro PC-3 cells studied. Results indicated a 40 % decrease in colony number for cells incubated with the decapeptide (SEQ ID NO: 3) at a concentration of 1 µg/ml. A decrease in colony number of up to 60 % was observed for the decapeptide (SEQ ID NO: 3) at a concentration of 10 µg/ml.

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EXAMPLE 5

DNA FRAGMENTATION ASSAY

Cell apoptosis result in DNA fragmentation can be evaluated by the presence of a DNA ladder visualized when DNA is run on a 1.2 % agarose gel. DNA ladder assay (apoptosis assay) was performed following exposure of PC-3 to various concentrations of the polypeptides for 72 hours. The polypeptides that were used in this particular experiment are polypeptide 7-21 (SEQ ID NO: 4), polypeptide PCK3145 (SEQ ID NO: 5) and polypeptide 76-94 (SEQ ID NO: 6). Visualization of DNA isolated and run on 1.2 % agarose gel, demonstrated that every polypeptides tested induced a DNA laddering effect characteristic of apoptosis. This effect was especially evident following treatment with PCK3145 (SEQ ID NO: 5), which is illustrated by figure 7. Lane 1 of the gel illustrated in figure 7 represents a lambda HindIII digest standard. Lane 2 of the gel illustrated in figure 7 represents DNA laddering effect obtained for doxorubicin-treated cells. Lane 3 of the gel illustrated in figure 7 represents DNA laddering effect obtained for cells incubated with 40 µg of nPSP94. Lane 4 of the gel illustrated in figure 7 represents DNA laddering effect obtained for cells incubated with 20 µg of nPSP94. Lane 5 of the gel illustrated in figure 7 represents DNA laddering effect obtained for cells incubated with 22.5 µM of PCK3145 (SEQ ID NO: 5). Lane 6 of the gel illustrated in figure 7 represents DNA laddering effect obtained for cells incubated with 45 µM of PCK3145 (SEQ ID NO: 5).

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EXAMPLE 6

APOPTOSIS ASSAY BY ELISA PLUS

5 The three polypeptides (SEQ ID NO: 4, SEQ ID NO: 5 and SEQ ID NO 6) and native PSP94 used here as a positive control were tested in ELISA plus assay to measure cell death through apoptosis. Briefly, the ELISA plus assay is a sandwich enzyme immunoassay able to measure mono- and oligonucleosomes present in the cytoplasmic fraction of cell lysate using two antibodies, one directed against DNA and the
10 other directed against histones. The apoptotic cell death is characterized by activation of endogenous endonucleases (e.g., calcium- and magnesium-dependant), which cleave double-stranded DNA at the most accessible internucleosomal linker region, generating mono- and oligonucleosomes. The enrichment of mono- and
15 oligonucleosomes in the cytoplasm of the apoptotic cells is due to the fact that DNA degradation occurs several hours before plasma membrane breakdown.

Four thousand cells were seeded in Costar 96 well cell
20 culture flat bottom plates in RPMI supplemented media containing 50 µg/ml of bovine serum albumin (BSA) and 0.1 µM FeSO₄. The PC-3 cells were treated with various concentrations (22.5 µM to 90 µM) of polypeptides for 72 hours. Apoptosis assay was done as per manufacturer's instructions using the ApopTag kit (Boehringer
25 Mannheim).

Results presented in figure 8, indicate a dose dependent increase in the apoptotic cell death effect was observed for every polypeptides used (SEQ ID NO: 4, SEQ ID NO: 5 and SEQ ID NO 6).
30 Polypeptide PCK3145 (SEQ ID NO: 5) was more potent than the other polypeptides at 90 µM concentration (Figure 8).

EXAMPLE 7

35 INHIBITION OF CELL-GROWTH BY PSP94 POLYPEPTIDES
(Figures 9 to 11)

Biological activity of the polypeptides as set forth in SEQ ID NO: 4, SEQ ID NO: 5 and SEQ ID NO 6 was determined by their growth
40 inhibitory effect on human prostate cancer cells PC-3. Native PSP94, rHuPSP94, polypeptide 22-36 and PB111 polypeptide (scrambled polypeptide) were also included in this experiment as controls. Cell proliferation assay was performed on either PC-3 cells or normal fibroblasts (used here as control) using the MTS/PMS kit (Promega).

Four thousand cells (Figure 9 and 10) or three thousand (figure 11) cells were seeded in Costar 96 well cell culture flat bottom plates in RPMI supplemented media containing 50 µg/ml of bovine serum albumin (BSA) and 0.1 µM FeSO₄. In addition, monitoring of cell morphology was also performed.

Results of these experiments are shown in figures 9 to 11. No cell inhibitory effect was observed following incubation of fibroblasts with various polypeptide concentrations (from 10 to 90 µM) for 72 hours (Figure 9). However, a significant growth inhibition was observed for polypeptides as set forth in SEQ ID NO: 4 and SEQ ID NO: 6 and more importantly with polypeptide PCK3145 (SEQ ID NO: 5) (Figure 10). Another experiment was performed using PCK3145 and polypeptide 22-36 at various concentrations on PC-3 cells, grown in OPTI-MEM media. In figures 9 to 11, the percentage of growth inhibition given for treated cells is evaluated relative to non-treated control cells for which a value of 100 % cell survival is given.

EXAMPLES 8 & 9

IN-VIVO EXPERIMENTS (Figures 12 & 13)

Studies MLL-1 and MLL-2 were performed as follows; on day 0, male Copenhagen rats were injected subcutaneously with 5×10^5 Mat LyLu cells per rat. These cells were derived from cultures of Mat LyLu cell line grown in RPMI media containing 10 % (v/v) of fetal calf serum in logarithmic phase of growth. Cells were harvested from the culture flasks by trypsinization, were centrifuged at 1200 rotation per minute (rpm) and washed three times with Hanks balanced salt solution (HBSS). Following washing, cells were counted and adjusted to a concentration of 5×10^6 cells/ml in HBSS. A 0.1 ml volume of tumor cell inoculum containing 5×10^5 cells was administered subcutaneously into the flank region of each rat. Three days after tumor cell implantation (i.e., inoculation), animals were treated daily by a subcutaneous injection of the desired polypeptide until day 13.

Experiments illustrated in figure 12 show the anti-tumor efficacy validation of rHuPSP94 against Mat LyLu (MLL) tumor implanted in nude mice (Protocol based on S. Garde et al.; The Prostate, 22: 225-233, 1993).

For study MLL-1 (Figure 12), tumor-implanted nude mice were separated in different groups, each receiving various amount of rHuPSP94 or control reagents. The different groups used in these experiments are illustrated below. Each group contained 8 mice.

5

Group 1: Negative control: PBS subcutaneously (s.c.)

Group 2: Positive control: Doxorubicin at 5mg/kg intraveanously (i.v.) single bolus on day 3

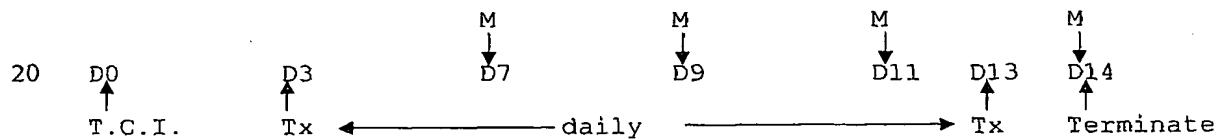
Group 3: rHuPSP94 at 1µg/kg/day (s.c.)

10 Group 4: rHuPSP94 at 10µg/kg/day (s.c.)

Group 5: rHuPSP94 at 100µg/kg/day (s.c.)

A schematic of inoculation is illustrated below;

15 (Tumor cell implantation (T.C.I.), treatment (Tx), measurement (M), day (D)).



25

Experiments illustrated in study MLL-2 show the anti-tumor efficacy validation of rHuPSP94 against Mat Ly Lu (MLL) tumor implanted in severe combined immunodeficiency (SCID) mice (Protocol based on S. Garde et al.; The Prostate, 22: 225-233, 1993).

30

For study MLL-2 (figure 13), tumor-implanted Scid mice were separated in different groups each receiving various amounts of rHuPSP94 or control reagents. The different groups used in these experiments are illustrated below. Each group contained 8 mice.

35

Group 1: Negative control: PBS (s.c.)

Group 2: Positive control: Doxorubicin at 5mg/kg i.v. single bolus on day 3

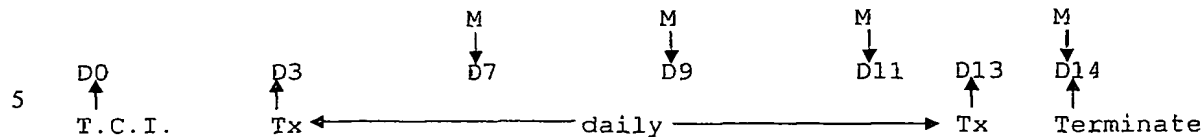
Group 3: rHuPSP94 at 1µg/kg/day (s.c.)

40 Group 4: rHuPSP94 at 10µg/kg/day (s.c.)

Group 5: rHuPSP94 at 100µg/kg/day (s.c.)

A schematic of inoculation is illustrated below;

45 (Tumor cell implantation (T.C.I.), treatment (Tx), measurement (M), day (D)).



10 Results of those two studies indicate a difference in tumor size and growth in Nude vs SCID mice. The tumors grew slower and were smaller in SCID mice. This may be due to some specific factors controlling tumor growth in this mouse strain. Results also show a significant tumor reduction in mice injected with Doxorubicin (positive control). For example, tumor weight reduction in Nude mice (study MLL-1) injected with Doxorubicin was 48 % ($p=0.006$) (p values measured by unpaired Student's t -test at $p<0.05$ as cut-off limit). Tumor weight reduction in SCID mice (study MLL-2) inoculated with Doxorubicin was 82 % ($p=0.002$) (p values measured by unpaired Student's t -test at $p<0.05$ as cut-off limit). Results indicate also a significant tumor reduction in mice treated with rHuPSP94 at a concentration of 1 $\mu\text{g/kg/day}$. For example, tumor weight reduction in Nude mice (study MLL-1) treated with rHuPSP94 at a concentration of 1 $\mu\text{g/kg/day}$ was 26 % ($p=0.042$) (p values measured by unpaired Student's t -test at $p<0.05$ as cut-off limit). Tumor weight reduction in SCID mice (study MLL-2) treated with rHuPSP94 at a concentration of 1 $\mu\text{g/kg/day}$ was 65 % ($p=0.010$) (p values measured by unpaired Student's t -test at $p<0.05$ as cut-off limit).

EXAMPLE 10

IN-VIVO EXPERIMENT USING PC-3 CELL LINE

(Figure 14)

35 PC-3 human prostate tumor was obtained from ATCC (ATCC 1435). PC-3 cells were grown in RPMI media containing 10 % (v/v) of fetal calf serum and were harvested in the logarithmic phase of growth by trypsinization. Cells were centrifuged at 1200 rotation per minute (rpm) and washed three times with Hanks balanced salt solution (HBSS). Following washing, cells were counted and adjusted to a concentration of 1×10^7 cells/ml in HBSS. A 0.1 ml volume of tumor cell inoculum containing 1×10^6 cells was administered subcutaneously into the two opposite flank region of each Nude mouse (Nu/Nu, BALB/c background). Tumor growth was monitored for approximately 18 days. Once tumor growth has been established (volume of tumor reached a volume of 50 mm^3) treatment

with rHuPSP94 (SEQ ID NO: 2) was initiated and was performed once a day for 14 days by the subcutaneous route. Based on the assigned treatment groups illustrated in table 4.

5 TABLE 4

Treatment group	Test control articles	Dose Level ($\mu\text{g/kg/day}$)	Dose concentration ($\mu\text{g/mg}$)	No. of animal
1 Negative control	PBS	0	0	8
2 Positive control	Doxorubicin	5000	2500	8
3	rHuPSP94	1	0.5	8
4	rHuPSP94	10	5	8
5	rHuPSP94	100	50	8
6	rHuPSP94	1000	500	8

10 Results of this experiment (Figure 14) demonstrated tumor growth reduction in the group of mice treated with rHuPSP94 at a dosage level of 1 $\mu\text{g/kg}$ body weight per day. This reduction was similar to that observed for Doxorubicin (given at 5 mg/kg/day) which is a chemotherapeutic agent used as reference gold standard.

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EXAMPLE 11

IN-VIVO EXPERIMENT USING PC-3 CELL LINE

(Figures 15-17)

20

PC-3 human prostate tumor (ATCC 1435) obtained from ATCC was implanted bilaterally into nude mice and tumor growth was monitored for approximately 18 days. PC-3 cells were injected once subcutaneously into each flank of the mice. Once tumor growth has been established (i.e., volume of tumor reached 0.25 to 0.50 cm^3) the treatment with decapeptide (SEQ ID NO: 3), native PSP94 (SEQ ID NO: 1) and control scrambled polypeptide PB111 was initiated and was performed once a day for 14 days by the subcutaneous route based on the treatment groups (randomly assigned) illustrated in table 5.

25

30

TABLE 5

Treatment groups	Test and control articles	Dose Level ($\mu\text{g/kg/day}$)	Dose concentration ($\mu\text{g/mg}$)	No. of animal
1 (Negative control)	PBS	0	0	4
3	Decapeptide (SEQ ID NO: 3)	1	0.5	4
4	Decapeptide (SEQ ID NO: 3)	10	5	4
5	Decapeptide (SEQ ID NO: 3)	100	50	4
6	Decapeptide (SEQ ID NO: 3)	1000	500	4
7	Native PSP94 (SEQ ID NO: 1)	1	0.5	4
8	Native PSP94 (SEQ ID NO: 1)	10	5	4
10	Native PSP94 (SEQ ID NO: 1)	100	50	4
11	Native PSP94 (SEQ ID NO: 1)	1000	500	4
12	Scrambled polypeptide (PB111)	1	0.5	4
13	Scrambled polypeptide (PB111)	10	5	4
14	Scrambled polypeptide (PB111)	100	50	4
15	Scrambled polypeptide (PB111)	1000	500	4

Figure 15 represents results obtained for tumor-implanted nude mice treated with the decapeptide (SEQ ID NO: 3) compared to a non-treated control. Figure 16 represents results obtained for tumor-implanted nude mice treated with scrambled polypeptide PB111 compared to a non-treated control. Figure 17 represents results obtained for tumor-implanted nude mice treated with native PSP94 (SEQ ID NO: 1) compared to a non-treated control. Results of these experiments (Figures 15-17) indicate a significant ($p < 0.05$) tumor growth reduction in mice treated with the decapeptide (SEQ ID NO: 3) at a dosage level of 10 $\mu\text{g/kg}$ body weight per day.

EXAMPLE 12
MANUFACTURING AND PREPARATION OF POLYPEPTIDES

5 PSP94 derived polypeptides including PCK3145 (SEQ ID NO: 5) were synthesized using the FMOC and BOC solid phase polypeptide synthesis method (Merrifield, B., Science, 232: 341-347, 1986). Polypeptides were analyzed in order to determine their identity by Mass Spectral Analysis. Polypeptide samples were analyzed using the
10 PerSeptive Biosystems (Framingham, MA), with Voyager-DE MALDI-TOF mass spectrometer using 337 nm light from a nitrogen laser. About 50 scans were averaged for each analysis. A sample from the native PSP94 was also analyzed under similar conditions for comparison. Polypeptides were weighed on a Mettler AE 163 micro-balance. The
15 measurements were to nearest 0.1 mg. The polypeptides were reconstituted in 10 mM PBS pH 7.3 to a final concentration of 1 and 5 mg/ml. The polypeptides dissolved relatively well and were filter sterilized through a 0.2 μ m syringe filter. Aliquots of 2 ml/tube were made and stored at -80 °C.

20 The pH of the polypeptides was measured after reconstitution to ensure that possible differences in pH would not be a factor of variation. The pH values of each solution were taken at three concentrations: neat, 100 μ g/ml and 12.5 μ g/ml. The pH range was
25 approximately from 7.0 to 7.5. This did not make a significant difference in the outcome of the test as cells survive very well within this pH range. To change the concentrations to molar values, the approximate volume of the 1 mg/ml stocks were diluted in PBS pH 7.3. All stocks were made to contain 450 μ M polypeptide solutions.
30 When fresh stocks of polypeptide were to be reconstituted, it was done directly to 450 μ M concentration in PBS pH 7.3.

35 After our initial screening and confirmation of the inhibitory activity of the polypeptide on the growth of the PC-3 cells, a GMP manufactured polypeptide was tested. This polypeptide was weighed and dissolved in PBS and 2 mg/ml stock solution was prepared, sterile filtered through a 0.2 μ m syringe filter and stored at in -80 °C.

EXAMPLE 13

EFFECT OF PCK3145 ON IN-VITRO PC-3 CELLS

5 (MTS ASSAY (Figures 18-21))

PCK3145, manufactured as set forth in example 12, was evaluated as a lead candidate product in tumor growth inhibition.

10

The biological activity of PCK3145 was determined by its growth inhibitory effect on the human prostate cancer cell line PC-3 using the MTS/PMS kit (Promega). This assay measures the mitochondrial activity of the live cells. The basic principle of this method involves the fact that the mitochondrial enzymes of the live cells metabolize the MTS/PMS dyes forming a brown colored precipitate which can be measured as optical density (OD) by absorption at 490 nm in a spectrophotometer. Therefore, the OD values are proportional to the number of living cells.

20

In addition, a visual observation of the cells was also done to check the cell morphology, which could also be indicative of cell growth. The following conditions for MTS assay were used: PC-3 (ATCC, Lot AT06), passage number $n \geq 70$, cell line adapted to grow in serum-free OPTI-MEM and in RPMI supplemented with BSA (50 $\mu\text{g/ml}$) and Ferrous Sulfate (0.1 μM), continuous exposure for up to 72 hours without changing media (i.e., adding PCK3145 at 2X concentration directly to wells and diluting it 1:2 to 1X to minimize cell manipulation and avoid detachment). As indicated in Figure 18, PCK3145 was assessed at the following concentrations: 12.5, 25, 50, 100, 200, 300 and 400 $\mu\text{g/ml}$ on PC-3 cells (ATCC) grown in supplemented media. The MTS tests were repeated 5 times and a dose dependent inhibitory effect on the growth of PC-3 cells was consistently reproducible demonstrating approximately 40 % cell growth inhibition at the highest PCK3145 concentration of 400 $\mu\text{g/ml}$.

35

With the availability of GMP (good manufacturing practice) grade polypeptide the MTS assays were repeated to check the reproducibility and cytotoxicity against PC-3 cells. In parallel PC-3 cells were also treated with the native PSP94 as a reference positive control and with no treatment (negative control, i.e., cont.). Figure 19 shows the results of the MTS assay where 4000 cells were seeded and exposed to PCK3145 (GMP grade) for 48 hours. A 30 % growth inhibitory effect was observed following treatment with

40

PCK3145 at 500 µg/ml. This effect was increased to approximately 40 % after 72 hours of exposure (Figure 20). In a repeat experiment a 48 hours exposure to the polypeptide at 500 µg/ml resulted in only 20-22 % growth inhibition, however this effect increased to 30 % after 72 hours exposure (Figure 21). Despite assay to assay variability reflected by the state of cell growth in vitro, polypeptide PCK3145 exhibited a significant cell growth inhibition.

EXAMPLE 14

EFFECT OF PCK3145 ON IN-VITRO PC-3 CELLS [³H]-THYMIDINE UPTAKE ASSAY (Figures 22-24)

[³H]-Thymidine uptake assay involves [³H]-Thymidine incorporation into cellular DNA of actively proliferating cells. [³H]-Thymidine uptake assay measures the proliferative index of the cells versus the MTS assay, which quantifies the number of lived cells following treatment. The anti-proliferative effects of PCK3145 and two other synthetic polypeptides derived from the amino and carboxy terminus ends of PSP94 (SEQ ID NO: 4 and NO: 6, respectively) as well as the decapeptide (SEQ ID NO: 3) previously shown to mimic the biological action of native PSP94 were assessed in [³H]-Thymidine uptake assay on PC-3 cells. Two separate experiments were conducted with GMP-grade PCK3145.

As shown in the Figures 22 and 23, polypeptide PCK3145 exhibited a significant proliferation inhibition activity reflected in the percentage of [³H]-Thymidine uptake. In the first experiment, a reduction of nearly 40 % in [³H]-Thymidine uptake was observed at PCK3145 concentration of 200 µg/ml. In the second experiment, although a two fold higher concentration of the PCK3145 was used (i.e., 400 µg/ml) only a 25 % inhibition was observed. Despite assay to assay variation the overall degree of proliferative inhibitory effect against PC-3 cell was markedly evident with the GMP grade material. Treatment of PC-3 cells with the native PSP94 used as a positive reference standard, exhibited a significant dose dependent reduction in cell proliferation with almost 50 % reduction in the [³H]-Thymidine uptake following 72 hours exposure (Figure 24).

EXAMPLE 15
IN VITRO EFFECT OF PCK3145 ON PC-3 CELLS
(APOPTOSIS-Figure 25)

5 Apoptosis of PC-3 cells, following a 72 hours exposure to PCK3145 at 500 µg/ml concentration, was evaluated in supplemented media by DNA fragmentation assay. Doxorubicin was used as a reference positive control. Untreated cells and PCK3145-treated cells were
10 harvested and the DNA was isolated. Isolated DNA was run on a 1.2 % agarose gel containing Ethidium Bromide (EtBr). As shown in Figure 25 treatment of PC-3 cells with polypeptide PKC3145 resulted in DNA fragmentation evidenced by the ladder formation seen for fragmented DNA. Lane 1 of the gel illustrated in figure 25 represents the DNA
15 marker (100 base pair DNA ladder). Lane 2 of the gel illustrated in figure 25 represents a control of untreated PC-3 cells. Lane 3 of the gel illustrated in figure 25 represents DNA laddering effect observed for cells treated with doxorubicin at a concentration of 2 µg/ml. Lane 4 of the gel illustrated in figure 25 represents DNA laddering
20 effect observed for cells treated with PCK3145 (SEQ ID NO: 5).

25
EXAMPLE 16
IN VIVO EXPERIMENTS USING HUMAN PC-3 PROSTATE CANCER CELL LINE
(Figures 26-27)

30 Studies PC3-6 and PC3-12 (Figures 26 - 27) are consecutive group experiments designed to characterize the in vivo activity of PCK3145 in the human PC-3 prostate cancer nude mouse xenograft model and to explore relationships between dose, route and schedule of administration and the efficacy parameters of tumor growth (volume).

35 PC-3 cells harvested in mid-log phase were inoculated at 5×10^6 cells per mice via the subcutaneous route in the mice's back area. Tumors grown from this inoculum were excised at approximately day 32 to 35 post-tumor implantation (p.t.i) when tumor volume reached 200-300 mm³ (i.e., cu mm). The necrotic tissue was removed
40 and the viable tumor mass cut into small pieces (approximately 1 to 3 mm³) were implanted SC in the flank region at two opposite sites of the mouse. Treatment with various concentrations of PCK3145 was initiated at day 3 post-tumor implantation (p.t.i) and was continued daily for 21 days. Subcutaneous injections were done below tumor
45 growth sites. Intra-peritoneal injections were performed in the

abdominal region. Intra-venous injections were performed via the lateral tail vein. The experiment was terminated 24 hours after the last treatment. Tumor measurements were taken at Days 11, 14, 16, 18, 20, 22 and 24 post-tumor implantation (p.t.i). Tumor volumes were calculated according to formula ($axb^2 \times 0.5$), where a - is the length of the long diameter, and b-is the width of the perpendicular small diameter.

Study No: PC3-6 illustrates the efficacy of PCK3145, injected subcutaneously, in tumor growth retardation in Nude mice, which have received PC-3 implants. Mice were separated in different group each receiving various amounts of PCK3145 (SEQ ID NO: 5) or control reagents. The different groups used in these experiments are illustrated in table 6 below. Each group contained 10 mice. Doxorubicin was administered as single bolus intra-venous injection on days 3 and 11 post-tumor implantation (p.t.i).

TABLE 6

Treatment group	Test and control articles	Dose Level ($\mu\text{g/kg/day}$)	No. of animals	No. of tumors
1 Negative control	PBS	0	10	20
2 Positive control	Doxorubicin	10000	10	20
3	PCK3145	0.1	10	20
4	PCK3145	1	10	20
5	PCK3145	10	10	20

20

Results of this study (Figure 26) demonstrated a significant PC-3 tumor growth retardation following treatment with PCK3145 at 10 $\mu\text{g/kg/day}$. This anti-tumor effect was evidenced by a statistically significant decrease in percentage of tumor growth observed at days 11, 14, 16, 18, 21 and 24 after tumor implantation with respective p-values ranging from $p=0.001$ to 0.002 , in comparison to the control PBS-treated group (p values measured by unpaired Student's t-test at $p<0.05$ as cut-off limit). Doxorubicin, a potent chemotherapeutic agent, was used as reference gold standard and demonstrated a highly significant anti-tumor therapeutic effect. ANOVA analysis of variance, Dunnett's test, Kruskal-Wallis and Dunn's test analysis of data confirmed statistical significance of the observed anti-tumor effect.

35

Study No: PC3-12 illustrates the efficacy of PCK3145 in tumor growth retardation in Nude mice, which have received PC-3 implants. Mice were separated in different group each receiving various amounts of PCK3145 (SEQ ID NO: 5) or control reagents. PCK3145 was injected either through intra-venous or intra-peritoneal route. The different groups used in these experiments are illustrated in table 7 below. Each group contained 9 mice.

TABLE 7

Treatment groups	Test and control articles	Dose level ($\mu\text{g/kg/day}$)	No. of animals	No. of tumors
1 Negative control	PBS	0	9	18
2	PCK3145 IV	10	9	18
3	PCK3145 IV	100	9	18
4	PCK3145 IV	500	9	18
5	PCK3145 IV	1000	9	18
6	PCK3145 IP	100	9	18
7	PCK3145 IP	1000	9	18

Results of this experiment (Figure 27) demonstrated a significant tumor growth retardation following treatment with PCK3145 at 100 $\mu\text{g/kg/day}$ via the intra-venous route. This effect was statistically significant at days 13, 17 and 20 after tumor implantation when compared by Student's t-test (p-values were $p=0.005$, 0.025 and 0.011 , respectively for each time-point) (p values measured by unpaired Student's t-test at $p<0.05$ as cut-off limit). No significant anti-tumor effect was observed following PCK3145 treatment at the other dosage levels of 10, 500 and 1000 $\mu\text{g/kg/day}$ injected via the intra-venous route. However a trend towards significance was observed following treatment with 500 and 1000 $\mu\text{g/kg/day}$ doses of PCK3145. Treatment of mice with PCK3145 at 100 and 1000 $\mu\text{g/kg/day}$ administered via the intra-peritoneal route showed a similar tumor growth retardation trend with statistically less significant difference observed at day 13 p.t.i ($p=0.056$) (p values measured by unpaired Student's t-test at $p<0.05$ as cut-off limit) at the highest dose of 1000 $\mu\text{g/kg/day}$ (Figure 27).

During the course of experimentation using the human PC-3 prostate cancer nude mouse xenograft model, results obtained have

suggested that subcutaneous PCK3145 injection of mice at a site (i.e., scruff of the neck) distant from tumor site, might not be efficacious enough and will unlikely may unlikely result in an anti-tumor effect, at least in the experimental conditions tested (doses of PCK3145 tested: 10 µg/kg/day and 100 µg/kg/day). The use of the scruff of the neck as a subcutaneous injection site represents an optimal site for immune response induction rather than a route for therapeutic product administration and as such, selection of this site is expected to be a sub-optimal site for tumor efficacy evaluation.

EXAMPLE 17

IN VIVO EXPERIMENTS USING DUNNING RAT MAT LY LU PROSTATE CANCER

LINE

(Figures 28-30)

Anti-tumor efficacy evaluation of PCK3145 against Mat Ly Lu (MLL) tumor implanted in Copenhagen rats was performed. (Protocol based on S. Garde et al.; The Prostate, 22: 225-233, 1993). Mat LyLu tumor cells were harvested in mid-log phase from the culture flasks by trypsinization, were centrifuged at 1200 rotation per minute (rpm) and washed three times with Hanks balanced salt solution (HBSS). Following washing, cells were counted and adjusted to a concentration of 5×10^6 cells/ml in HBSS. A 0.1 ml volume of tumor cell inoculum containing 5×10^5 cells was administered subcutaneously into the flank region of each rat. Treatment started at day 3 post-tumor implantation (p.t.i) by local subcutaneous injection (i.e., in the shaved back area just below tumor implantation site) of various PCK3145 concentrations. This treatment was continued daily for 16 days. Experiments were terminated 24 hours after the last treatment. Tumor measurements were taken at days 7, 9, 11, 14, 16 and 18. Tumor volumes are calculated according to formula ($a \times b^2 \times 0.5$), where a - is the length of the long diameter, b-width of the perpendicular small diameter. At day 19 tumors of individual rats were excised and weighed.

Study No: MLL-5 illustrates the efficacy of PCK3145 (SEQ ID NO: 5) compared with polypeptide 7-21 (SEQ ID NO: 4) and polypeptide 76-94 (SEQ ID NO: 6) in tumor growth retardation in Copenhagen rats, which have received Mat Ly Lu implants. Mice were separated in different groups, each receiving various amount of PCK3145 (SEQ ID NO: 5) or control reagents. PCK3145 was injected through the

subcutaneous route. The different groups used in these experiments are illustrated in table 8 below. Each group contained 8 mice.

5 TABLE 8

Treatment groups	Test and control articles	Dose Level ($\mu\text{g/kg/day}$)	No. of animals	No. of tumors
1 Negative control	PBS	0	8	8
2	Polypeptide 7-21	10	8	8
3	Polypeptide 7-21	1	8	8
4	PCK3145	10	8	8
5	PCK3145	1	8	8
6	Polypeptide 76-94	10	8	8
7	Polypeptide 76-94	1	8	8

10 Results of this study (Figure 28) demonstrated a significant anti-tumor effect following administration of PCK3145 at 10 $\mu\text{g/kg/day}$. This was evidenced by a significant tumor volume reduction at days 11 ($p=0.006$), 13 ($p=0.00001$), 16 ($p=0.002$) and 18 ($p=0.004$), post-tumor cell implantation compared to control PBS-
 15 treated group (p values measured by unpaired Student's t -test at $p<0.05$ as cut-off limit). No significant effect was detectable following PCK3145 treatment at 1 $\mu\text{g/kg/day}$. It was of interest to note that the amino-terminus polypeptide 7-21 also demonstrated comparable anti-tumor effect, which was also observed in the PC-3
 20 nude mouse xenograft model, indicating the possibility of an overlapping active site between the N-terminus and the central regions of the PSP94 protein. This was evidenced by a significant tumor volume reduction observed at day 13 ($p=0.05$), 16 ($p=0.00005$), and 18 ($p=0.01$) in mice treated with polypeptide 7-21 (p values
 25 measured by unpaired Student's t -test at $p<0.05$ as cut-off limit).

Study No: MLL-6 illustrates the efficacy of PCK3145 (SEQ ID NO: 5) in tumor growth retardation in Copenhagen rats, which have received Mat Ly Lu implants. Mice were separated in different group
 30 each receiving various amounts of PCK3145 (SEQ ID NO: 5) or control reagents. PCK3145 was injected through the subcutaneous route. The

different groups used in these experiments are illustrated in table 9 below. Each group contained 8 mice. Doxorubicin was administered as single bolus via intra-venous injection on day 3 p.t.i.

5

TABLE 9

Treatment groups	Test and control articles	Dose level ($\mu\text{g/kg/day}$)	No. of animals	No. of tumors
1 (Negative control)	PBS	0	8	8
2	Doxorubicin	5000	8	8
3	PCK3145	10	8	8
4	PCK3145	100	8	8
5	Scrambled polypeptide	10	8	8
6	Scrambled polypeptide	100	8	8

10

Results of this study (Figures 29 and 30) demonstrated a significant dose-dependent anti-tumor effect following administration of PCK3145 at 10 and 100 $\mu\text{g/kg/day}$. This was evidenced by a significant tumor volume reduction (31 % over control) following PCK3145 treatment especially with 100 $\mu\text{g/kg/day}$ at days 14, 16 and 18 post-tumor cell implantation (Figure 29). The p-value versus negative control-treated group (i.e., scrambled polypeptide (PB111)) was highly significant at $p=0.0000062$ (p values measured by unpaired Student's t-test at $p<0.05$ as cut-off limit). A moderate extent of growth retardation (marginal statistical significance at $p=0.03$ versus control PBS-treated group) was also observed following treatment with scrambled polypeptide at a concentration of 100 $\mu\text{g/kg/day}$ (Figure 29) (p values measured by unpaired Student's t-test at $p<0.05$ as cut-off limit). Doxorubicin treatment was highly significant resulting in over 80 % reduction in tumor volumes. This anti-tumor effect of PCK3145 at 100 $\mu\text{g/kg/day}$ was also reproduced following analysis of the tumor weights data. As shown in figure 30, (tumor weight data) a significant reduction in tumor weights ($p=0.0003$) was observed on day 18 p.t.i (p values measured by unpaired Student's t-test at $p<0.05$ as cut-off limit). This represented a 34 % reduction in tumor mass, a 20 gram difference between the control (56.6 g) and PCK3145-treated at 100 $\mu\text{g/kg/day}$ group (37.2 g). This difference in tumor weights was also

statistically significant when it was compared to the tumor weights of the control scrambled polypeptide-treated rats given the same dose of 100 µg/kg/day ($p=0.003$) (p values measured by unpaired Student's t-test at $p<0.05$ as cut-off limit). Comparison of the scrambled polypeptide treated tumor weights with that of control PBS-untreated tumor weights was not statistically significant ($p=0.06$) (p values measured by unpaired Student's t-test at $p<0.05$ as cut-off limit).

EXAMPLE 18

EFFICACY OF PCK3145 AND TAXOTERE COMBINATION TREATMENT

In order to test for the efficacy of combination treatment, in tumor growth retardation, PCK3145 and taxotere (i.e., docetaxel) were co-administered in Nude mice previously inoculated with PC-3 tumor cells. Mice were separated in different groups each receiving PCK3145 alone or PCK3145 in combination with taxotere (administered by separate routes) or control reagent (i.e., PBS). In this experiment, the combination treatment was initiated against relatively large tumor burdens. Tumors were allowed to grow beyond the 50 to 60 mm³ size at which PCK3145 treatment usually becomes inefficient. PCK3145 was injected through intravenous route every other day for 28 days starting from day 1 when 50 to 60 mm³ size subcutaneous tumors were apparent. Taxotere was injected by intra-peritoneal route at a sub-optimal concentration of 2 mg/kg on days 4 and 11 after subcutaneous tumors were evident. The different groups used in this experiment are illustrated in table 10 below. Each group contained 11 mice.

TABLE 10

Treatment groups	Test and control articles	Dose level (µg/kg)	No. of animals	No of tumors
1. Negative control	PBS	0	11	11
2. Positive control	Taxotere	2000	11	11
3.	PCK3145	100	11	11
4.	PCK3145 + taxotere	100 + 2000	11	11

Results of this experiment (Figure 31) demonstrate a significant tumor growth retardation following combination treatment of PCK3145 and taxotere. This effect is statistically significant at days 19 and 22 post-tumor cell inoculation when compared by Student's t-test ($p=0.02$ at day 19 and $p=0.047$ at day 22), (p-values are measured by unpaired Student's t-test at $p<0.05$ as a cut-off limit) and was markedly better than taxotere administered alone at the same dose of 2 mg/kg (suboptimal dose).

All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference. The citation of any publication is for its disclosure prior to the filing date and should not be construed as an admission that the present invention is not entitled to antedate such publication by virtue of prior invention.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it is readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

SEQUENCE LISTING

5 (1) GENERAL INFORMATION:

(i) APPLICANT: PROCYON BIOPHARMA INC.

10 (ii) TITLE OF INVENTION: PHARMACEUTICAL PREPARATIONS AND
METHODS FOR INHIBITING TUMORS

(iii) NUMBER OF SEQUENCES: 92

(iv) CORRESPONDENCE ADDRESS:

15 (A) ADDRESSEE: BROULLETTE KOSIE
(B) STREET: 1100 RENE-LESVEQUE BLVD WEST
(C) PROV/STATE: QUEBEC
(D) COUNTRY: CANADA
20 (E) POSTAL/ZIP CODE: H3B 5C9

(v) COMPUTER READABLE FORM:

25 (A) MEDIUM TYPE: FLOPPY DISK
(B) COMPUTER: IBM PC COMPATIBLE
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: ASCII (TEXT)

(vi) CURRENT APPLICATION DATA:

30 (A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

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35 (A) APPLICATION NUMBER: 2,321,256
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(2) INFORMATION FOR SEQ ID NO: 1:

50 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 94 AMINO ACIDS
(B) TYPE: AMINO ACIDS
(C) STRANDEDNESS: SINGLE
(D) TOPOLOGY: LINEAR

55 (ii) MOLECULE TYPE: PROTEIN

(iii) HYPOTHETICAL:

(iv) ANTI-SENSE:

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(A) ORGANISM:

60 (vii) IMMEDIATE SOURCE:

(viii) POSITION IN GENOME

(A) CHROMOSOME/SEGMENT:

(B) MAP POSITION:

(C) UNITS:

65 (ix) FEATURE:

(A) NAME/KEY:
 (B) LOCATION:
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20 Ser Cys Tyr Phe Ile Pro Asn Glu Gly Val Pro Gly Asp Ser Thr Arg
 1 5 10 15
 Lys Cys Met Asp Leu Lys Gly Asn Lys His Pro Ile Asn Ser Glu Trp
 20 25 30
 25 Gln Thr Asp Asn Cys Glu Thr Cys Thr Cys Tyr Glu Thr Glu Ile Ser
 35 40 45
 Cys Cys Thr Leu Val Ser Thr Pro Val Gly Tyr Asp Lys Asp Asn Cys
 30 50 55 60
 Gln Arg Ile Phe Lys Lys Glu Asp Cys Lys Tyr Ile Val Val Glu Lys
 65 70 75 80
 35 Lys Asp Pro Lys Lys Thr Cys Ser Val Ser Glu Trp Ile Ile
 85 90

40 (2) INFORMATION FOR SEQ ID NO: 2:
 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 102 AMINO ACIDS
 (B) TYPE: AMINO ACIDS
 (C) STRANDEDNESS: SINGLE
 (D) TOPOLOGY: LINEAR
 45 (ii) MOLECULE TYPE: PROTEIN

(vi) ORIGINAL SOURCE:
 50 (A) ORGANISM:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

55 Glu Ala Glu Ala Tyr Val Glu Phe Ser Cys Tyr Phe Ile Pro Asn Glu
 1 5 10 15
 Gly Val Pro Gly Asp Ser Thr Arg Lys Cys Met Asp Leu Lys Gly Asn
 20 25 30
 60 Lys His Pro Ile Asn Ser Glu Trp Gln Thr Asp Asn Cys Glu Thr Cys
 35 40 45
 Thr Cys Tyr Glu Thr Glu Ile Ser Cys Cys Thr Leu Val Ser Thr Pro
 65 50 55 60

Val Gly Tyr Asp Lys Asp Asn Cys Gln Arg Ile Phe Lys Lys Glu Asp
65 70 75 80

5 Cys Lys Tyr Ile Val Val Glu Lys Lys Asp Pro Lys Lys Thr Cys Ser
85 90 95

Val Ser Glu Trp Ile Ile
100

10

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 AMINO ACIDS
(B) TYPE: AMINO ACIDS
(C) STRANDEDNESS: SINGLE
(D) TOPOLOGY: LINEAR

15

(ii) MOLECULE TYPE: PROTEIN

20

(vi) ORIGINAL SOURCE:

(A) ORGANISM:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

25 Tyr Thr Cys Ser Val Ser Glu Pro Gly Ile
1 5 10

30

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 AMINO ACIDS
(B) TYPE: AMINO ACIDS
(C) STRANDEDNESS: SINGLE
(D) TOPOLOGY: LINEAR

35

(ii) MOLECULE TYPE: PROTEIN

(vi) ORIGINAL SOURCE:

40

(A) ORGANISM:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

45 Asn Glu Gly Val Pro Gly Asp Ser Thr Arg Lys Cys Met Asp Leu
1 5 10 15

50

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 AMINO ACIDS
(B) TYPE: AMINO ACIDS
(C) STRANDEDNESS: SINGLE
(D) TOPOLOGY: LINEAR

55

(ii) MOLECULE TYPE: PROTEIN

(vi) ORIGINAL SOURCE:

60

(A) ORGANISM:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

65 Glu Trp Gln Thr Asp Asn Cys Glu Thr Cys Thr Cys Tyr Glu Thr
1 5 10 15

(2) INFORMATION FOR SEQ ID NO: 6:

5 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 19 AMINO ACIDS
 (B) TYPE: AMINO ACIDS
 (C) STRANDEDNESS: SINGLE
 (D) TOPOLOGY: LINEAR

10 (ii) MOLECULE TYPE: PROTEIN

(vi) ORIGINAL SOURCE:

(A) ORGANISM:

15 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

Ile Val Val Glu Lys Lys Asp Pro Lys Lys Thr Cys Ser Val Ser Glu
 1 5 10 15

20 Trp Ile Ile

(2) INFORMATION FOR SEQ ID NO: 7:

25 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 26
 (B) TYPE: NUCLEOTIDES
 (C) STRANDEDNESS: SINGLE
 (D) TOPOLOGY: LINEAR

30 (ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

35

GGAAGAATT CTCATGCTAT TTCATA

26

(2) INFORMATION FOR SEQ ID NO: 8:

40 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 21
 (B) TYPE: NUCLEOTIDES
 (C) STRANDEDNESS: SINGLE
 (D) TOPOLOGY: LINEAR

45 (ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM:

50 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

TGGATATCTG CAGAATTCGG C

21

55 2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH:
 (B) TYPE: NUCLEOTIDES
 (C) STRANDEDNESS: SINGLE
 (D) TOPOLOGY: LINEAR

60 (ii) MOLECULE TYPE:

(vi) ORIGINAL SOURCE:
(A) ORGANISM:

5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

TCATGCTATT TCATACCTAA TGAGGGAGTT CCAGGAGATT CAACCAGGAA ATGCATGGAT 60
CTCAAAGGAA ACAAACACCC AATAAACTCG GAGTGGCAGA CTGACAACTG TGAGACATGC 120
10 ACTTGCTACG AAACAGAAAT TTCATGTTGC ACCCTTGTTT CTACACCTGT GGGTTATGAC 180
AAAGACAACT GCCAAAGAAT CTTCAAGAAG GAGGACTGCA AGTATATCGT GGTGGAGAAG 240
15 AAGGACCCAA AAAAGACCTG TTCTGTCAGT GAATGGATAA TCTAA 285

2) INFORMATION FOR SEQ ID NO: 10:

20 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16
(B) TYPE: AMINO ACID
(C) STRANDEDNESS: SINGLE
(D) TOPOLOGY: LINEAR

25 (ii) MOLECULE TYPE:

(vi) ORIGINAL SOURCE:
(A) ORGANISM:

30 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

Glu Trp Gln Thr Asp Asn Cys Glu Thr Cys Thr Cys Tyr Glu Thr Glu
1 5 10 15

35

2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17
(B) TYPE: AMINO ACID
40 (C) STRANDEDNESS: SINGLE
(D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE:

45 (vi) ORIGINAL SOURCE:
(A) ORGANISM:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

50 Glu Trp Gln Thr Asp Asn Cys Glu Thr Cys Thr Cys Tyr Glu Thr Glu
1 5 10 15

Ile

2) INFORMATION FOR SEQ ID NO: 12:

55 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18
(B) TYPE: AMINO ACID
(C) STRANDEDNESS: SINGLE
(D) TOPOLOGY: LINEAR

60 (ii) MOLECULE TYPE:

(vi) ORIGINAL SOURCE:

(A) ORGANISM:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

5 Glu Trp Gln Thr Asp Asn Cys Glu Thr Cys Thr Cys Tyr Glu Thr Glu
1 5 10 15
Ile Ser

10 2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 19

(B) TYPE: AMINO ACID

(C) STRANDEDNESS: SINGLE

15 (D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE:

(vi) ORIGINAL SOURCE:

(A) ORGANISM:

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

Glu Trp Gln Thr Asp Asn Cys Glu Thr Cys Thr Cys Tyr Glu Thr Glu
1 5 10 15
25 Ile Ser Cys

30 2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: AMINO ACID

(C) STRANDEDNESS: SINGLE

35 (D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE:

(vi) ORIGINAL SOURCE:

(A) ORGANISM:

40

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

Glu Trp Gln Thr Asp Asn Cys Glu Thr Cys Thr Cys Tyr Glu Thr Glu
1 5 10 15
45 Ile Ser Cys Cys
20

50

2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21

(B) TYPE: AMINO ACID

55 (C) STRANDEDNESS: SINGLE

(D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE:

(vi) ORIGINAL SOURCE:

(A) ORGANISM:

60

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

Glu Trp Gln Thr Asp Asn Cys Glu Thr Cys Thr Cys Tyr Glu Thr Glu
 1 5 10 15

Ile Ser Cys Cys Thr
 5 20

2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 22
 (B) TYPE: AMINO ACID
 (C) STRANDEDNESS: SINGLE
 (D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE:

(vi) ORIGINAL SOURCE:
 (A) ORGANISM:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

Glu Trp Gln Thr Asp Asn Cys Glu Thr Cys Thr Cys Tyr Glu Thr Glu
 1 5 10 15

Ile Ser Cys Cys Thr Leu
 25 20

2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 23
 (B) TYPE: AMINO ACID
 (C) STRANDEDNESS: SINGLE
 (D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE:

(vi) ORIGINAL SOURCE:
 (A) ORGANISM:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

Glu Trp Gln Thr Asp Asn Cys Glu Thr Cys Thr Cys Tyr Glu Thr Glu
 1 5 10 15

Ile Ser Cys Cys Thr Leu Val
 45 20

2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 24
 (B) TYPE: AMINO ACID
 (C) STRANDEDNESS: SINGLE
 (D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE:

(vi) ORIGINAL SOURCE:
 (A) ORGANISM:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

Glu Trp Gln Thr Asp Asn Cys Glu Thr Cys Thr Cys Tyr Glu Thr Glu
 1 5 10 15

Ile Ser Cys Cys Thr Leu Val Ser
 65 20

2) INFORMATION FOR SEQ ID NO: 19:

5 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 25
 (B) TYPE: AMINO ACID
 (C) STRANDEDNESS: SINGLE
 (D) TOPOLOGY: LINEAR

10 (ii) MOLECULE TYPE:

(vi) ORIGINAL SOURCE:
 (A) ORGANISM:

15 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

Glu Trp Gln Thr Asp Asn Cys Glu Thr Cys Thr Cys Tyr Glu Thr Glu
 1 5 10 15
 20 Ile Ser Cys Cys Thr Leu Val Ser Thr
 20 25

2) INFORMATION FOR SEQ ID NO: 20:

25 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 26
 (B) TYPE: AMINO ACID
 (C) STRANDEDNESS: SINGLE
 (D) TOPOLOGY: LINEAR

30 (ii) MOLECULE TYPE:

(vi) ORIGINAL SOURCE:
 (A) ORGANISM:

35 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

Glu Trp Gln Thr Asp Asn Cys Glu Thr Cys Thr Cys Tyr Glu Thr Glu
 1 5 10 15
 40 Ile Ser Cys Cys Thr Leu Val Ser Thr Pro
 20 25

2) INFORMATION FOR SEQ ID NO: 21:

45 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 27
 (B) TYPE: AMINO ACID
 (C) STRANDEDNESS: SINGLE
 (D) TOPOLOGY: LINEAR

50 (ii) MOLECULE TYPE:

(vi) ORIGINAL SOURCE:
 (A) ORGANISM:

55 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

Glu Trp Gln Thr Asp Asn Cys Glu Thr Cys Thr Cys Tyr Glu Thr Glu
 1 5 10 15
 60 Ile Ser Cys Cys Thr Leu Val Ser Thr Pro Val
 20 25

2) INFORMATION FOR SEQ ID NO: 22:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 28
- (B) TYPE: AMINO ACID
- (C) STRANDEDNESS: SINGLE
- (D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE:

(vi) ORIGINAL SOURCE:

- (A) ORGANISM:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

Glu Trp Gln Thr Asp Asn Cys Glu Thr Cys Thr Cys Tyr Glu Thr Glu
1 5 10 15
Ile Ser Cys Cys Thr Leu Val Ser Thr Pro Val Gly
20 25

2) INFORMATION FOR SEQ ID NO: 23:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 29
- (B) TYPE: AMINO ACID
- (C) STRANDEDNESS: SINGLE
- (D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE:

(vi) ORIGINAL SOURCE:

- (A) ORGANISM:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

Glu Trp Gln Thr Asp Asn Cys Glu Thr Cys Thr Cys Tyr Glu Thr Glu
1 5 10 15
Ile Ser Cys Cys Thr Leu Val Ser Thr Pro Val Gly Tyr
20 25

2) INFORMATION FOR SEQ ID NO: 24:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30
- (B) TYPE: AMINO ACID
- (C) STRANDEDNESS: SINGLE
- (D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE:

(vi) ORIGINAL SOURCE:

- (A) ORGANISM:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

Glu Trp Gln Thr Asp Asn Cys Glu Thr Cys Thr Cys Tyr Glu Thr Glu
1 5 10 15
Ile Ser Cys Cys Thr Leu Val Ser Thr Pro Val Gly Tyr Asp
20 25 30

2) INFORMATION FOR SEQ ID NO: 25:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 31
 (B) TYPE: AMINO ACID
 (C) STRANDEDNESS: SINGLE
 (D) TOPOLOGY: LINEAR

5 (ii) MOLECULE TYPE:

(vi) ORIGINAL SOURCE:

(A) ORGANISM:

10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

Glu Trp Gln Thr Asp Asn Cys Glu Thr Cys Thr Cys Tyr Glu Thr Glu
 1 5 10 15

15 Ile Ser Cys Cys Thr Leu Val Ser Thr Pro Val Gly Tyr Asp Lys
 20 25 30

20 2) INFORMATION FOR SEQ ID NO: 26:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 32
 (B) TYPE: AMINO ACID
 (C) STRANDEDNESS: SINGLE
 (D) TOPOLOGY: LINEAR

25 (ii) MOLECULE TYPE:

(vi) ORIGINAL SOURCE:

(A) ORGANISM:

30 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

Glu Trp Gln Thr Asp Asn Cys Glu Thr Cys Thr Cys Tyr Glu Thr Glu
 1 5 10 15

35 Ile Ser Cys Cys Thr Leu Val Ser Thr Pro Val Gly Tyr Asp Lys Asp
 20 25 30

40 2) INFORMATION FOR SEQ ID NO: 27:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 33
 (B) TYPE: AMINO ACID
 (C) STRANDEDNESS: SINGLE
 (D) TOPOLOGY: LINEAR

45 (ii) MOLECULE TYPE:

(vi) ORIGINAL SOURCE:

(A) ORGANISM:

50 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:

Glu Trp Gln Thr Asp Asn Cys Glu Thr Cys Thr Cys Tyr Glu Thr Glu
 1 5 10 15

55 Ile Ser Cys Cys Thr Leu Val Ser Thr Pro Val Gly Tyr Asp Lys Asp
 20 25 30

Asn

60 2) INFORMATION FOR SEQ ID NO: 28:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 34

(B) TYPE: AMINO ACID
 (C) STRANDEDNESS: SINGLE
 (D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE:

5

(vi) ORIGINAL SOURCE:

(A) ORGANISM:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 28:

10 Glu Trp Gln Thr Asp Asn Cys Glu Thr Cys Thr Cys Tyr Glu Thr Glu
 1 5 10 15

Ile Ser Cys Cys Thr Leu Val Ser Thr Pro Val Gly Tyr Asp Lys Asp
 20 25 30

15

Asn Cys

2) INFORMATION FOR SEQ ID NO: 29:

20 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 35

(B) TYPE: AMINO ACID

(C) STRANDEDNESS: SINGLE

(D) TOPOLOGY: LINEAR

25

(ii) MOLECULE TYPE:

(vi) ORIGINAL SOURCE:

(A) ORGANISM:

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 29:

Glu Trp Gln Thr Asp Asn Cys Glu Thr Cys Thr Cys Tyr Glu Thr Glu
 1 5 10 15

35 Ile Ser Cys Cys Thr Leu Val Ser Thr Pro Val Gly Tyr Asp Lys Asp
 20 25 30

Asn Cys Gln
 35

40

2) INFORMATION FOR SEQ ID NO: 30:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 36

(B) TYPE: AMINO ACID

45

(C) STRANDEDNESS: SINGLE

(D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE:

(vi) ORIGINAL SOURCE:

50

(A) ORGANISM:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 30:

Glu Trp Gln Thr Asp Asn Cys Glu Thr Cys Thr Cys Tyr Glu Thr Glu
 1 5 10 15

55

Ile Ser Cys Cys Thr Leu Val Ser Thr Pro Val Gly Tyr Asp Lys Asp
 20 25 30

Asn Cys Gln Arg
 35

60

2) INFORMATION FOR SEQ ID NO: 31:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 37
 (B) TYPE: AMINO ACID
 (C) STRANDEDNESS: SINGLE
 (D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE:

(vi) ORIGINAL SOURCE:

- (A) ORGANISM:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 31:

Glu Trp Gln Thr Asp Asn Cys Glu Thr Cys Thr Cys Tyr Glu Thr Glu
 1 5 10 15

Ile Ser Cys Cys Thr Leu Val Ser Thr Pro Val Gly Tyr Asp Lys Asp
 20 25 30

Asn Cys Gln Arg Ile
 35

2) INFORMATION FOR SEQ ID NO: 32:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 38
 (B) TYPE: AMINO ACID
 (C) STRANDEDNESS: SINGLE
 (D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE:

(vi) ORIGINAL SOURCE:

- (A) ORGANISM:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 32:

Glu Trp Gln Thr Asp Asn Cys Glu Thr Cys Thr Cys Tyr Glu Thr Glu
 1 5 10 15

Ile Ser Cys Cys Thr Leu Val Ser Thr Pro Val Gly Tyr Asp Lys Asp
 20 25 30

Asn Cys Gln Arg Ile Phe
 35

2) INFORMATION FOR SEQ ID NO: 33:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 39
 (B) TYPE: AMINO ACID
 (C) STRANDEDNESS: SINGLE
 (D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE:

(vi) ORIGINAL SOURCE:

- (A) ORGANISM:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 33:

Glu Trp Gln Thr Asp Asn Cys Glu Thr Cys Thr Cys Tyr Glu Thr Glu
 1 5 10 15

Ile Ser Cys Cys Thr Leu Val Ser Thr Pro Val Gly Tyr Asp Lys Asp
 20 25 30

Asn Cys Gln Arg Ile Phe Lys
 35

2) INFORMATION FOR SEQ ID NO: 34:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 40
- (B) TYPE: AMINO ACID
- (C) STRANDEDNESS: SINGLE
- (D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE:

(vi) ORIGINAL SOURCE:

(A) ORGANISM:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 34:

Glu Trp Gln Thr Asp Asn Cys Glu Thr Cys Thr Cys Tyr Glu Thr Glu
 1 5 10 15
 Ile Ser Cys Cys Thr Leu Val Ser Thr Pro Val Gly Tyr Asp Lys Asp
 20 25 30
 Asn Cys Gln Arg Ile Phe Lys Lys
 35 40

2) INFORMATION FOR SEQ ID NO: 35:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 41
- (B) TYPE: AMINO ACID
- (C) STRANDEDNESS: SINGLE
- (D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE:

(vi) ORIGINAL SOURCE:

(A) ORGANISM:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 35:

Glu Trp Gln Thr Asp Asn Cys Glu Thr Cys Thr Cys Tyr Glu Thr Glu
 1 5 10 15
 Ile Ser Cys Cys Thr Leu Val Ser Thr Pro Val Gly Tyr Asp Lys Asp
 20 25 30
 Asn Cys Gln Arg Ile Phe Lys Lys Glu
 35 40

2) INFORMATION FOR SEQ ID NO: 36:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 42
- (B) TYPE: AMINO ACID
- (C) STRANDEDNESS: SINGLE
- (D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE:

(vi) ORIGINAL SOURCE:

(A) ORGANISM:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 36:

Glu Trp Gln Thr Asp Asn Cys Glu Thr Cys Thr Cys Tyr Glu Thr Glu
 1 5 10 15
 5 Ile Ser Cys Cys Thr Leu Val Ser Thr Pro Val Gly Tyr Asp Lys Asp
 20 25 30
 Asn Cys Gln Arg Ile Phe Lys Lys Glu Asp
 35 40
 10

2) INFORMATION FOR SEQ ID NO: 37:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 43
 (B) TYPE: AMINO ACID
 (C) STRANDEDNESS: SINGLE
 (D) TOPOLOGY: LINEAR
 (ii) MOLECULE TYPE:

(vi) ORIGINAL SOURCE:
 (A) ORGANISM:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 37:

Glu Trp Gln Thr Asp Asn Cys Glu Thr Cys Thr Cys Tyr Glu Thr Glu
 1 5 10 15
 25 Ile Ser Cys Cys Thr Leu Val Ser Thr Pro Val Gly Tyr Asp Lys Asp
 20 25 30
 30 Asn Cys Gln Arg Ile Phe Lys Lys Glu Asp Cys
 35 40

2) INFORMATION FOR SEQ ID NO: 38:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 44
 (B) TYPE: AMINO ACID
 (C) STRANDEDNESS: SINGLE
 (D) TOPOLOGY: LINEAR
 (ii) MOLECULE TYPE:

(vi) ORIGINAL SOURCE:
 (A) ORGANISM:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 38:

Glu Trp Gln Thr Asp Asn Cys Glu Thr Cys Thr Cys Tyr Glu Thr Glu
 1 5 10 15
 50 Ile Ser Cys Cys Thr Leu Val Ser Thr Pro Val Gly Tyr Asp Lys Asp
 20 25 30
 Asn Cys Gln Arg Ile Phe Lys Lys Glu Asp Cys Lys
 35 40

55

2) INFORMATION FOR SEQ ID NO: 39:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 45
 (B) TYPE: AMINO ACID
 (C) STRANDEDNESS: SINGLE
 (D) TOPOLOGY: LINEAR

60

(ii) MOLECULE TYPE:

(vi) ORIGINAL SOURCE:

(A) ORGANISM:

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 39:

10 Glu Trp Gln Thr Asp Asn Cys Glu Thr Cys Thr Cys Tyr Glu Thr Glu
 1 5 10 15
 Ile Ser Cys Cys Thr Leu Val Ser Thr Pro Val Gly Tyr Asp Lys Asp
 20 25 30
 15 Asn Cys Gln Arg Ile Phe Lys Lys Glu Asp Cys Lys Tyr
 35 40 45

2) INFORMATION FOR SEQ ID NO: 40:

(i) SEQUENCE CHARACTERISTICS:

20 (A) LENGTH: 46
 (B) TYPE: AMINO ACID
 (C) STRANDEDNESS: SINGLE
 (D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE:

25

(vi) ORIGINAL SOURCE:

(A) ORGANISM:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 40:

30 Glu Trp Gln Thr Asp Asn Cys Glu Thr Cys Thr Cys Tyr Glu Thr Glu
 1 5 10 15
 Ile Ser Cys Cys Thr Leu Val Ser Thr Pro Val Gly Tyr Asp Lys Asp
 20 25 30
 35 Asn Cys Gln Arg Ile Phe Lys Lys Glu Asp Cys Lys Tyr Ile
 35 40 45

40 2) INFORMATION FOR SEQ ID NO: 41:

(i) SEQUENCE CHARACTERISTICS:

45 (A) LENGTH: 47
 (B) TYPE: AMINO ACID
 (C) STRANDEDNESS: SINGLE
 (D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE:

(vi) ORIGINAL SOURCE:

(A) ORGANISM:

50

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 41:

Glu Trp Gln Thr Asp Asn Cys Glu Thr Cys Thr Cys Tyr Glu Thr Glu
 1 5 10 15
 55 Ile Ser Cys Cys Thr Leu Val Ser Thr Pro Val Gly Tyr Asp Lys Asp
 20 25 30
 Asn Cys Gln Arg Ile Phe Lys Lys Glu Asp Cys Lys Tyr Ile Val
 35 40 45

60

2) INFORMATION FOR SEQ ID NO: 42:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 48
 (B) TYPE: AMINO ACID
 (C) STRANDEDNESS: SINGLE
 (D) TOPOLOGY: LINEAR
 (ii) MOLECULE TYPE:
 (vi) ORIGINAL SOURCE:
 (A) ORGANISM:
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 42:

Glu Trp Gln Thr Asp Asn Cys Glu Thr Cys Thr Cys Tyr Glu Thr Glu
 1 5 10 15
 Ile Ser Cys Cys Thr Leu Val Ser Thr Pro Val Gly Tyr Asp Lys Asp
 20 25 30
 Asn Cys Gln Arg Ile Phe Lys Lys Glu Asp Cys Lys Tyr Ile Val Val
 35 40 45

2) INFORMATION FOR SEQ ID NO: 43:
 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 49
 (B) TYPE: AMINO ACID
 (C) STRANDEDNESS: SINGLE
 (D) TOPOLOGY: LINEAR
 (ii) MOLECULE TYPE:
 (vi) ORIGINAL SOURCE:
 (A) ORGANISM:
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 43:

Glu Trp Gln Thr Asp Asn Cys Glu Thr Cys Thr Cys Tyr Glu Thr Glu
 1 5 10 15
 Ile Ser Cys Cys Thr Leu Val Ser Thr Pro Val Gly Tyr Asp Lys Asp
 20 25 30
 Asn Cys Gln Arg Ile Phe Lys Lys Glu Asp Cys Lys Tyr Ile Val Val
 35 40 45
 Glu

2) INFORMATION FOR SEQ ID NO: 44:
 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 50
 (B) TYPE: AMINO ACID
 (C) STRANDEDNESS: SINGLE
 (D) TOPOLOGY: LINEAR
 (ii) MOLECULE TYPE:
 (vi) ORIGINAL SOURCE:
 (A) ORGANISM:
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 44:

Glu Trp Gln Thr Asp Asn Cys Glu Thr Cys Thr Cys Tyr Glu Thr Glu
 1 5 10 15

Ile Ser Cys Cys Thr Leu Val Ser Thr Pro Val Gly Tyr Asp Lys Asp
 20 25 30

5 Asn Cys Gln Arg Ile Phe Lys Lys Glu Asp Cys Lys Tyr Ile Val Val
 35 40 45

Glu Lys
 50

10

2) INFORMATION FOR SEQ ID NO: 45:

 (i) SEQUENCE CHARACTERISTICS:

 (A) LENGTH: 51

 (B) TYPE: AMINO ACID

 (C) STRANDEDNESS: SINGLE

 (D) TOPOLOGY: LINEAR

 (ii) MOLECULE TYPE:

20 (vi) ORIGINAL SOURCE:

 (A) ORGANISM:

 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 45:

25 Glu Trp Gln Thr Asp Asn Cys Glu Thr Cys Thr Cys Tyr Glu Thr Glu
 1 5 10 15

Ile Ser Cys Cys Thr Leu Val Ser Thr Pro Val Gly Tyr Asp Lys Asp
 20 25 30

30 Asn Cys Gln Arg Ile Phe Lys Lys Glu Asp Cys Lys Tyr Ile Val Val
 35 40 45

Glu Lys Lys
 50

35

2) INFORMATION FOR SEQ ID NO: 46:

 (i) SEQUENCE CHARACTERISTICS:

 (A) LENGTH: 52

 (B) TYPE: AMINO ACID

 (C) STRANDEDNESS: SINGLE

 (D) TOPOLOGY: LINEAR

 (ii) MOLECULE TYPE:

45 (vi) ORIGINAL SOURCE:

 (A) ORGANISM:

 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 46:

50

Glu Trp Gln Thr Asp Asn Cys Glu Thr Cys Thr Cys Tyr Glu Thr Glu
 1 5 10 15

Ile Ser Cys Cys Thr Leu Val Ser Thr Pro Val Gly Tyr Asp Lys Asp
 20 25 30

55 Asn Cys Gln Arg Ile Phe Lys Lys Glu Asp Cys Lys Tyr Ile Val Val
 35 40 45

Glu Lys Lys Asp
 50

60

2) INFORMATION FOR SEQ ID NO: 47:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 53
- (B) TYPE: AMINO ACID
- (C) STRANDEDNESS: SINGLE
- (D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE:

(vi) ORIGINAL SOURCE:

- (A) ORGANISM:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 47:

Glu Trp Gln Thr Asp Asn Cys Glu Thr Cys Thr Cys Tyr Glu Thr Glu
 1 5 10 15
 Ile Ser Cys Cys Thr Leu Val Ser Thr Pro Val Gly Tyr Asp Lys Asp
 20 25 30
 Asn Cys Gln Arg Ile Phe Lys Lys Glu Asp Cys Lys Tyr Ile Val Val
 35 40 45
 Glu Lys Lys Asp Pro
 50

2) INFORMATION FOR SEQ ID NO: 48:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 54
- (B) TYPE: AMINO ACID
- (C) STRANDEDNESS: SINGLE
- (D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE:

(vi) ORIGINAL SOURCE:

- (A) ORGANISM:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 48:

Glu Trp Gln Thr Asp Asn Cys Glu Thr Cys Thr Cys Tyr Glu Thr Glu
 1 5 10 15
 Ile Ser Cys Cys Thr Leu Val Ser Thr Pro Val Gly Tyr Asp Lys Asp
 20 25 30
 Asn Cys Gln Arg Ile Phe Lys Lys Glu Asp Cys Lys Tyr Ile Val Val
 35 40 45
 Glu Lys Lys Asp Pro Lys
 50

2) INFORMATION FOR SEQ ID NO: 49:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 55
- (B) TYPE: AMINO ACID
- (C) STRANDEDNESS: SINGLE
- (D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE:

(vi) ORIGINAL SOURCE:

- (A) ORGANISM:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 49:

Glu Trp Gln Thr Asp Asn Cys Glu Thr Cys Thr Cys Tyr Glu Thr Glu
1 5 10 15
5 Ile Ser Cys Cys Thr Leu Val Ser Thr Pro Val Gly Tyr Asp Lys Asp
20 25 30
Asn Cys Gln Arg Ile Phe Lys Lys Glu Asp Cys Lys Tyr Ile Val Val
35 40 45
10 Glu Lys Lys Asp Pro Lys Lys
50 55

15 2) INFORMATION FOR SEQ ID NO: 50:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 56

(B) TYPE: AMINO ACID

(C) STRANDEDNESS: SINGLE

(D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE:

(vi) ORIGINAL SOURCE:

(A) ORGANISM:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 50:

Glu Trp Gln Thr Asp Asn Cys Glu Thr Cys Thr Cys Tyr Glu Thr Glu
1 5 10 15
30 Ile Ser Cys Cys Thr Leu Val Ser Thr Pro Val Gly Tyr Asp Lys Asp
20 25 30
Asn Cys Gln Arg Ile Phe Lys Lys Glu Asp Cys Lys Tyr Ile Val Val
35 35 40 45
Glu Lys Lys Asp Pro Lys Lys Thr
50 55

40 2) INFORMATION FOR SEQ ID NO: 51:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 57

(B) TYPE: AMINO ACID

(C) STRANDEDNESS: SINGLE

(D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE:

(vi) ORIGINAL SOURCE:

(A) ORGANISM:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 51:

Glu Trp Gln Thr Asp Asn Cys Glu Thr Cys Thr Cys Tyr Glu Thr Glu
1 5 10 15
55 Ile Ser Cys Cys Thr Leu Val Ser Thr Pro Val Gly Tyr Asp Lys Asp
20 25 30
60 Asn Cys Gln Arg Ile Phe Lys Lys Glu Asp Cys Lys Tyr Ile Val Val
35 40 45

Glu Lys Lys Asp Pro Lys Lys Thr Cys
50 55

- 5 2) INFORMATION FOR SEQ ID NO: 52:
 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 58
 (B) TYPE: AMINO ACID
 (C) STRANDEDNESS: SINGLE
 10 (D) TOPOLOGY: LINEAR
 (ii) MOLECULE TYPE:
 (vi) ORIGINAL SOURCE:
 (A) ORGANISM:
 15 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 52:

Glu Trp Gln Thr Asp Asn Cys Glu Thr Cys Thr Cys Tyr Glu Thr Glu
1 5 10 15
 20 Ile Ser Cys Cys Thr Leu Val Ser Thr Pro Val Gly Tyr Asp Lys Asp
20 25 30
 25 Asn Cys Gln Arg Ile Phe Lys Lys Glu Asp Cys Lys Tyr Ile Val Val
35 40 45
 Glu Lys Lys Asp Pro Lys Lys Thr Cys Ser
50 55

- 30 2) INFORMATION FOR SEQ ID NO: 53:
 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 59
 (B) TYPE: AMINO ACID
 35 (C) STRANDEDNESS: SINGLE
 (D) TOPOLOGY: LINEAR
 (ii) MOLECULE TYPE:
 (vi) ORIGINAL SOURCE:
 40 (A) ORGANISM:
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 53:

45 Glu Trp Gln Thr Asp Asn Cys Glu Thr Cys Thr Cys Tyr Glu Thr Glu
1 5 10 15
 Ile Ser Cys Cys Thr Leu Val Ser Thr Pro Val Gly Tyr Asp Lys Asp
20 25 30
 50 Asn Cys Gln Arg Ile Phe Lys Lys Glu Asp Cys Lys Tyr Ile Val Val
35 40 45
 Glu Lys Lys Asp Pro Lys Lys Thr Cys Ser Val
50 55

- 2) INFORMATION FOR SEQ ID NO: 54:
 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 60
 60 (B) TYPE: AMINO ACID

(C) STRANDEDNESS: SINGLE
 (D) TOPOLOGY: LINEAR
 (ii) MOLECULE TYPE:

5 (vi) ORIGINAL SOURCE:
 (A) ORGANISM:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 54:

10 Glu Trp Gln Thr Asp Asn Cys Glu Thr Cys Thr Cys Tyr Glu Thr Glu
 1 5 10 15
 Ile Ser Cys Cys Thr Leu Val Ser Thr Pro Val Gly Tyr Asp Lys Asp
 20 25 30
 15 Asn Cys Gln Arg Ile Phe Lys Lys Glu Asp Cys Lys Tyr Ile Val Val
 35 40 45
 20 Glu Lys Lys Asp Pro Lys Lys Thr Cys Ser Val Ser
 50 55 60

2) INFORMATION FOR SEQ ID NO: 55:

25 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 61
 (B) TYPE: AMINO ACID
 (C) STRANDEDNESS: SINGLE
 (D) TOPOLOGY: LINEAR

30 (ii) MOLECULE TYPE:

(vi) ORIGINAL SOURCE:
 (A) ORGANISM:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 55:

35 Glu Trp Gln Thr Asp Asn Cys Glu Thr Cys Thr Cys Tyr Glu Thr Glu
 1 5 10 15
 40 Ile Ser Cys Cys Thr Leu Val Ser Thr Pro Val Gly Tyr Asp Lys Asp
 20 25 30
 Asn Cys Gln Arg Ile Phe Lys Lys Glu Asp Cys Lys Tyr Ile Val Val
 35 40 45
 45 Glu Lys Lys Asp Pro Lys Lys Thr Cys Ser Val Ser Glu
 50 55 60

50 2) INFORMATION FOR SEQ ID NO: 56:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 62
 (B) TYPE: AMINO ACID
 (C) STRANDEDNESS: SINGLE
 (D) TOPOLOGY: LINEAR

55 (ii) MOLECULE TYPE:

(vi) ORIGINAL SOURCE:
 (A) ORGANISM:

60

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 56:

5 Glu Trp Gln Thr Asp Asn Cys Glu Thr Cys Thr Cys Tyr Glu Thr Glu
 1 5 10 15
 Ile Ser Cys Cys Thr Leu Val Ser Thr Pro Val Gly Tyr Asp Lys Asp
 20 25 30
 10 Asn Cys Gln Arg Ile Phe Lys Lys Glu Asp Cys Lys Tyr Ile Val Val
 35 40 45
 Glu Lys Lys Asp Pro Lys Lys Thr Cys Ser Val Ser Glu Trp
 50 55 60

15

2) INFORMATION FOR SEQ ID NO: 57:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 63
 (B) TYPE: AMINO ACID
 (C) STRANDEDNESS: SINGLE
 (D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE:

(vi) ORIGINAL SOURCE:

(A) ORGANISM:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 57:

30 Glu Trp Gln Thr Asp Asn Cys Glu Thr Cys Thr Cys Tyr Glu Thr Glu
 1 5 10 15
 Ile Ser Cys Cys Thr Leu Val Ser Thr Pro Val Gly Tyr Asp Lys Asp
 20 25 30
 35 Asn Cys Gln Arg Ile Phe Lys Lys Glu Asp Cys Lys Tyr Ile Val Val
 35 40 45
 Glu Lys Lys Asp Pro Lys Lys Thr Cys Ser Val Ser Glu Trp Ile
 50 55 60

40

2) INFORMATION FOR SEQ ID NO: 58:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 64
 (B) TYPE: AMINO ACID
 (C) STRANDEDNESS: SINGLE
 (D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE:

(vi) ORIGINAL SOURCE:

(A) ORGANISM:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 58:

55 Glu Trp Gln Thr Asp Asn Cys Glu Thr Cys Thr Cys Tyr Glu Thr Glu
 1 5 10 15
 Ile Ser Cys Cys Thr Leu Val Ser Thr Pro Val Gly Tyr Asp Lys Asp
 20 25 30

60

Asn Cys Gln Arg Ile Phe Lys Lys Glu Asp Cys Lys Tyr Ile Val Val
 35 40 45

5 Glu Lys Lys Asp Pro Lys Lys Thr Cys Ser Val Ser Glu Trp Ile Ile
 50 55 60

2) INFORMATION FOR SEQ ID NO: 59:

10 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 16
 (B) TYPE: AMINO ACID
 (C) STRANDEDNESS: SINGLE
 (D) TOPOLOGY: LINEAR

15 (ii) MOLECULE TYPE:

(vi) ORIGINAL SOURCE:

(A) ORGANISM:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 59:

20

Ser Glu Trp Gln Thr Asp Asn Cys Glu Thr Cys Thr Cys Tyr Glu Thr
 1 5 10 15

25 2) INFORMATION FOR SEQ ID NO: 60:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 17
 (B) TYPE: AMINO ACID
 (C) STRANDEDNESS: SINGLE
 (D) TOPOLOGY: LINEAR

30 (ii) MOLECULE TYPE:

(vi) ORIGINAL SOURCE:

(A) ORGANISM:

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 60:

Asn Ser Glu Trp Gln Thr Asp Asn Cys Glu Thr Cys Thr Cys Tyr Glu
 1 5 10 15

40

Thr

2) INFORMATION FOR SEQ ID NO: 61:

45 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 18
 (B) TYPE: AMINO ACID
 (C) STRANDEDNESS: SINGLE
 (D) TOPOLOGY: LINEAR

50 (ii) MOLECULE TYPE:

(vi) ORIGINAL SOURCE:

(A) ORGANISM:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 61:

55

Ile Asn Ser Glu Trp Gln Thr Asp Asn Cys Glu Thr Cys Thr Cys Tyr
 1 5 10 15

Glu Thr

2) INFORMATION FOR SEQ ID NO: 62:

5 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 19
 (B) TYPE: AMINO ACID
 (C) STRANDEDNESS: SINGLE
 (D) TOPOLOGY: LINEAR

10 (ii) MOLECULE TYPE:

(vi) ORIGINAL SOURCE:

(A) ORGANISM:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 62:

15

Pro Ile Asn Ser Glu Trp Gln Thr Asp Asn Cys Glu Thr Cys Thr Cys
 1 5 10 15

20 Tyr Glu Thr

2) INFORMATION FOR SEQ ID NO: 63:

25 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 20
 (B) TYPE: AMINO ACID
 (C) STRANDEDNESS: SINGLE
 (D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE:

30 (vi) ORIGINAL SOURCE:

(A) ORGANISM:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 63:

35 His Pro Ile Asn Ser Glu Trp Gln Thr Asp Asn Cys Glu Thr Cys Thr
 1 5 10 15

40 Cys Tyr Glu Thr
 20

2) INFORMATION FOR SEQ ID NO: 64:

45 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 21
 (B) TYPE: AMINO ACID
 (C) STRANDEDNESS: SINGLE
 (D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE:

50 (vi) ORIGINAL SOURCE:

(A) ORGANISM:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 64:

55 Lys His Pro Ile Asn Ser Glu Trp Gln Thr Asp Asn Cys Glu Thr Cys
 1 5 10 15

Thr Cys Tyr Glu Thr
 20

2) INFORMATION FOR SEQ ID NO: 65:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22
 (B) TYPE: AMINO ACID
 (C) STRANDEDNESS: SINGLE
 (D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE:

(vi) ORIGINAL SOURCE:

- (A) ORGANISM:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 65:

Asn Lys His Pro Ile Asn Ser Glu Trp Gln Thr Asp Asn Cys Glu Thr
 1 5 10 15
 Cys Thr Cys Tyr Glu Thr
 20

2) INFORMATION FOR SEQ ID NO: 66:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 23
 (B) TYPE: AMINO ACID
 (C) STRANDEDNESS: SINGLE
 (D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE:

(vi) ORIGINAL SOURCE:

- (A) ORGANISM:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 66:

Gly Asn Lys His Pro Ile Asn Ser Glu Trp Gln Thr Asp Asn Cys Glu
 1 5 10 15
 Thr Cys Thr Cys Tyr Glu Thr
 20

2) INFORMATION FOR SEQ ID NO: 67:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24
 (B) TYPE: AMINO ACID
 (C) STRANDEDNESS: SINGLE
 (D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE:

(vi) ORIGINAL SOURCE:

- (A) ORGANISM:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 67:

Lys Gly Asn Lys His Pro Ile Asn Ser Glu Trp Gln Thr Asp Asn Cys
 1 5 10 15
 Glu Thr Cys Thr Cys Tyr Glu Thr
 20

2) INFORMATION FOR SEQ ID NO: 68:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25
- (B) TYPE: AMINO ACID
- (C) STRANDEDNESS: SINGLE
- (D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE:

(vi) ORIGINAL SOURCE:

(A) ORGANISM:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 68:

15 Leu Lys Gly Asn Lys His Pro Ile Asn Ser Glu Trp Gln Thr Asp Asn
 1 5 10 15
 Cys Glu Thr Cys Thr Cys Tyr Glu Thr
 20 25

2) INFORMATION FOR SEQ ID NO: 69:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26
- (B) TYPE: AMINO ACID
- (C) STRANDEDNESS: SINGLE
- (D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE:

(vi) ORIGINAL SOURCE:

(A) ORGANISM:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 69:

35 Asp Leu Lys Gly Asn Lys His Pro Ile Asn Ser Glu Trp Gln Thr Asp
 1 5 10 15
 Asn Cys Glu Thr Cys Thr Cys Tyr Glu Thr
 20 25

2) INFORMATION FOR SEQ ID NO: 70:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27
- (B) TYPE: AMINO ACID
- (C) STRANDEDNESS: SINGLE
- (D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE:

(vi) ORIGINAL SOURCE:

(A) ORGANISM:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 70:

55 Met Asp Leu Lys Gly Asn Lys His Pro Ile Asn Ser Glu Trp Gln Thr
 1 5 10 15
 Asp Asn Cys Glu Thr Cys Thr Cys Tyr Glu Thr
 20 25

2) INFORMATION FOR SEQ ID NO: 71:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 28
 (B) TYPE: AMINO ACID
 (C) STRANDEDNESS: SINGLE
 (D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE:

(vi) ORIGINAL SOURCE:

(A) ORGANISM:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 71:

Cys Met Asp Leu Lys Gly Asn Lys His Pro Ile Asn Ser Glu Trp Gln
 1 5 10 15

Thr Asp Asn Cys Glu Thr Cys Thr Cys Tyr Glu Thr
 20 25

2) INFORMATION FOR SEQ ID NO: 72:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 29
 (B) TYPE: AMINO ACID
 (C) STRANDEDNESS: SINGLE
 (D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE:

(vi) ORIGINAL SOURCE:

(A) ORGANISM:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 72:

Lys Cys Met Asp Leu Lys Gly Asn Lys His Pro Ile Asn Ser Glu Trp
 1 5 10 15

Gln Thr Asp Asn Cys Glu Thr Cys Thr Cys Tyr Glu Thr
 20 25

2) INFORMATION FOR SEQ ID NO: 73:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30
 (B) TYPE: AMINO ACID
 (C) STRANDEDNESS: SINGLE
 (D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE:

(vi) ORIGINAL SOURCE:

(A) ORGANISM:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 73:

Arg Lys Cys Met Asp Leu Lys Gly Asn Lys His Pro Ile Asn Ser Glu
 1 5 10 15

Trp Gln Thr Asp Asn Cys Glu Thr Cys Thr Cys Tyr Glu Thr
 20 25 30

2) INFORMATION FOR SEQ ID NO: 74:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 31
 (B) TYPE: AMINO ACID
 (C) STRANDEDNESS: SINGLE
 (D) TOPOLOGY: LINEAR

5 (ii) MOLECULE TYPE:

(vi) ORIGINAL SOURCE:
 (A) ORGANISM:

10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 74:

Thr Arg Lys Cys Met Asp Leu Lys Gly Asn Lys His Pro Ile Asn Ser
 1 5 10 15

15 Glu Trp Gln Thr Asp Asn Cys Glu Thr Cys Thr Cys Tyr Glu Thr
 20 25 30

2) INFORMATION FOR SEQ ID NO: 75:

20 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 32
 (B) TYPE: AMINO ACID
 (C) STRANDEDNESS: SINGLE
 (D) TOPOLOGY: LINEAR

25 (ii) MOLECULE TYPE:

(vi) ORIGINAL SOURCE:
 (A) ORGANISM:

30 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 75:

Ser Thr Arg Lys Cys Met Asp Leu Lys Gly Asn Lys His Pro Ile Asn
 1 5 10 15

35 Ser Glu Trp Gln Thr Asp Asn Cys Glu Thr Cys Thr Cys Tyr Glu Thr
 20 25 30

2) INFORMATION FOR SEQ ID NO: 76:

40 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 33
 (B) TYPE: AMINO ACID
 (C) STRANDEDNESS: SINGLE
 (D) TOPOLOGY: LINEAR

45 (ii) MOLECULE TYPE:

(vi) ORIGINAL SOURCE:
 (A) ORGANISM:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 76:

50 Asp Ser Thr Arg Lys Cys Met Asp Leu Lys Gly Asn Lys His Pro Ile
 1 5 10 15

55 Asn Ser Glu Trp Gln Thr Asp Asn Cys Glu Thr Cys Thr Cys Tyr Glu
 20 25 30

Thr

2) INFORMATION FOR SEQ ID NO: 77:

60 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 34

(B) TYPE: AMINO ACID
 (C) STRANDEDNESS: SINGLE
 (D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE:

(vi) ORIGINAL SOURCE:

(A) ORGANISM:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 77:

10

Gly Asp Ser Thr Arg Lys Cys Met Asp Leu Lys Gly Asn Lys His Pro
 1 5 10 15

15

Ile Asn Ser Glu Trp Gln Thr Asp Asn Cys Glu Thr Cys Thr Cys Tyr
 20 25 30

Glu Thr

2) INFORMATION FOR SEQ ID NO: 78:

20

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 35

(B) TYPE: AMINO ACID

(C) STRANDEDNESS: SINGLE

(D) TOPOLOGY: LINEAR

25

(ii) MOLECULE TYPE:

(vi) ORIGINAL SOURCE:

(A) ORGANISM:

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 78:

Pro Gly Asp Ser Thr Arg Lys Cys Met Asp Leu Lys Gly Asn Lys His
 1 5 10 15

35

Pro Ile Asn Ser Glu Trp Gln Thr Asp Asn Cys Glu Thr Cys Thr Cys
 20 25 30

Tyr Glu Thr

35

40

2) INFORMATION FOR SEQ ID NO: 79:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 36

(B) TYPE: AMINO ACID

45

(C) STRANDEDNESS: SINGLE

(D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE:

(vi) ORIGINAL SOURCE:

50

(A) ORGANISM:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 79:

Val Pro Gly Asp Ser Thr Arg Lys Cys Met Asp Leu Lys Gly Asn Lys
 1 5 10 15

55

His Pro Ile Asn Ser Glu Trp Gln Thr Asp Asn Cys Glu Thr Cys Thr
 20 25 30

Cys Tyr Glu Thr

60

35

2) INFORMATION FOR SEQ ID NO: 80:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 37
 (B) TYPE: AMINO ACID
 (C) STRANDEDNESS: SINGLE
 (D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE:

(vi) ORIGINAL SOURCE:

- (A) ORGANISM:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 80:

Gly Val Pro Gly Asp Ser Thr Arg Lys Cys Met Asp Leu Lys Gly Asn
 1 5 10 15
 Lys His Pro Ile Asn Ser Glu Trp Gln Thr Asp Asn Cys Glu Thr Cys
 20 25 30
 Thr Cys Tyr Glu Thr
 35

2) INFORMATION FOR SEQ ID NO: 81:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 38
 (B) TYPE: AMINO ACID
 (C) STRANDEDNESS: SINGLE
 (D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE:

(vi) ORIGINAL SOURCE:

- (A) ORGANISM:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 81:

Glu Gly Val Pro Gly Asp Ser Thr Arg Lys Cys Met Asp Leu Lys Gly
 1 5 10 15
 Asn Lys His Pro Ile Asn Ser Glu Trp Gln Thr Asp Asn Cys Glu Thr
 20 25 30
 Cys Thr Cys Tyr Glu Thr
 35

2) INFORMATION FOR SEQ ID NO: 82:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 39
 (B) TYPE: AMINO ACID
 (C) STRANDEDNESS: SINGLE
 (D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE:

(vi) ORIGINAL SOURCE:

- (A) ORGANISM:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 82:

Asn Glu Gly Val Pro Gly Asp Ser Thr Arg Lys Cys Met Asp Leu Lys
 1 5 10 15

Gly Asn Lys His Pro Ile Asn Ser Glu Trp Gln Thr Asp Asn Cys Glu
 20 25 30

Thr Cys Thr Cys Tyr Glu Thr

5 35

2) INFORMATION FOR SEQ ID NO: 83:

10 (i) SEQUENCE CHARACTERISTICS:

 (A) LENGTH: 40
 (B) TYPE: AMINO ACID
 (C) STRANDEDNESS: SINGLE
 (D) TOPOLOGY: LINEAR

15 (ii) MOLECULE TYPE:

 (vi) ORIGINAL SOURCE:

 (A) ORGANISM:

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 83:

Pro Asn Glu Gly Val Pro Gly Asp Ser Thr Arg Lys Cys Met Asp Leu
 1 5 10 15

25 Lys Gly Asn Lys His Pro Ile Asn Ser Glu Trp Gln Thr Asp Asn Cys
 20 25 30

Glu Thr Cys Thr Cys Tyr Glu Thr

 35 40

30

2) INFORMATION FOR SEQ ID NO: 84:

 (i) SEQUENCE CHARACTERISTICS:

 (A) LENGTH: 41
 (B) TYPE: AMINO ACID
 (C) STRANDEDNESS: SINGLE
 (D) TOPOLOGY: LINEAR

35 (ii) MOLECULE TYPE:

 (vi) ORIGINAL SOURCE:

 (A) ORGANISM:

40

 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 84:

45 Ile Pro Asn Glu Gly Val Pro Gly Asp Ser Thr Arg Lys Cys Met Asp
 1 5 10 15

Leu Lys Gly Asn Lys His Pro Ile Asn Ser Glu Trp Gln Thr Asp Asn
 20 25 30

Cys Glu Thr Cys Thr Cys Tyr Glu Thr

50 35 40

2) INFORMATION FOR SEQ ID NO: 85:

55 (i) SEQUENCE CHARACTERISTICS:

 (A) LENGTH: 42
 (B) TYPE: AMINO ACID
 (C) STRANDEDNESS: SINGLE
 (D) TOPOLOGY: LINEAR

60 (ii) MOLECULE TYPE:

(vi) ORIGINAL SOURCE:

(A) ORGANISM:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 85:

5

Phe Ile Pro Asn Glu Gly Val Pro Gly Asp Ser Thr Arg Lys Cys Met
 1 5 10 15

10

Asp Leu Lys Gly Asn Lys His Pro Ile Asn Ser Glu Trp Gln Thr Asp
 20 25 30

Asn Cys Glu Thr Cys Thr Cys Tyr Glu Thr
 35 40

15

2) INFORMATION FOR SEQ ID NO: 86:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 43

(B) TYPE: AMINO ACID

(C) STRANDEDNESS: SINGLE

20

(D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE:

(vi) ORIGINAL SOURCE:

(A) ORGANISM:

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 86:

Tyr Phe Ile Pro Asn Glu Gly Val Pro Gly Asp Ser Thr Arg Lys Cys
 1 5 10 15

30

Met Asp Leu Lys Gly Asn Lys His Pro Ile Asn Ser Glu Trp Gln Thr
 20 25 30

Asp Asn Cys Glu Thr Cys Thr Cys Tyr Glu Thr
 35 40

35

2) INFORMATION FOR SEQ ID NO: 87:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 44

(B) TYPE: AMINO ACID

(C) STRANDEDNESS: SINGLE

(D) TOPOLOGY: LINEAR

40

(ii) MOLECULE TYPE:

(vi) ORIGINAL SOURCE:

(A) ORGANISM:

45

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 87:

Cys Tyr Phe Ile Pro Asn Glu Gly Val Pro Gly Asp Ser Thr Arg Lys
 1 5 10 15

50

Cys Met Asp Leu Lys Gly Asn Lys His Pro Ile Asn Ser Glu Trp Gln
 20 25 30

55

Thr Asp Asn Cys Glu Thr Cys Thr Cys Tyr Glu Thr
 35 40

2) INFORMATION FOR SEQ ID NO: 88:

60

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 45
 (C) TYPE: AMINO ACID
 (C) STRANDEDNESS: SINGLE
 (D) TOPOLOGY: LINEAR
 5 (ii) MOLECULE TYPE:
 (vi) ORIGINAL SOURCE:
 (A) ORGANISM:
 10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 88:

 Ser Cys Tyr Phe Ile Pro Asn Glu Gly Val Pro Gly Asp Ser Thr Arg
 1 5 10 15
 15 Lys Cys Met Asp Leu Lys Gly Asn Lys His Pro Ile Asn Ser Glu Trp
 20 25 30
 Gln Thr Asp Asn Cys Glu Thr Cys Thr Cys Tyr Glu Thr
 35 40 45

20

2) INFORMATION FOR SEQ ID NO: 89:

25 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 15
 (B) TYPE: AMINO ACID
 (C) STRANDEDNESS: SINGLE
 (D) TOPOLOGY: LINEAR
 (ii) MOLECULE TYPE:
 30 (ix) FEATURE :
 (A) NAME/KEY : Modified site
 (B) LOCATION : 1
 (D) OTHER INFORMATION : The residue in this
 35 position is either glutamic acid, asparagine, or
 aspartic acid.
 (ix) FEATURE :
 (A) NAME/KEY : Modified site
 (B) LOCATION : 4
 (D) OTHER INFORMATION : The residue in this position
 40 is either threonine, or serine.
 (ix) FEATURE :
 (A) NAME/KEY : Modified site
 (B) LOCATION : 6
 (D) OTHER INFORMATION : The residue in this position
 45 is either glutamic acid, asparagine, or aspartic
 acid.
 (ix) FEATURE :
 (A) NAME/KEY : Modified site
 (B) LOCATION : 8
 (D) OTHER INFORMATION : The residue in this position
 50 is either glutamic acid, asparagine, or aspartic
 acid.
 (ix) FEATURE :
 (A) NAME/KEY : Modified site
 (B) LOCATION : 9
 (D) OTHER INFORMATION : The residue in this position
 55 is either threonine, or serine.
 (ix) FEATURE :
 (A) NAME/KEY : Modified site
 (B) LOCATION : 11
 (D) OTHER INFORMATION : The residue in this position
 60 is either threonine, or serine.
 (ix) FEATURE :
 (A) NAME/KEY : Modified site

(B)LOCATION : 13
 (D)OTHER INFORMATION : The residue in this position
 is either tyrosine, or phenylalanine.

5 (ix) FEATURE :
 (A)NAME/KEY : Modified site
 (B)LOCATION : 14
 (D)OTHER INFORMATION : The residue in this position
 is either glutamic acid, asparagine, or aspartic
 acid.

10 (ix) FEATURE :
 (A)NAME/KEY : Modified site
 (B)LOCATION : 15
 (D)OTHER INFORMATION : The residue in this position
 is either threonine, or serine.

15 (vi)ORIGINAL SOURCE:
 (A) ORGANISM:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 89:

20

Xaa Trp Gln Xaa Asp Xaa Cys Xaa Xaa Cys Xaa Cys Xaa Xaa Xaa
 1 5 10 15

25 2) INFORMATION FOR SEQ ID NO: 90:
 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 30
 (B) TYPE: AMINO ACID
 (C) STRANDEDNESS: SINGLE
 (D) TOPOLOGY: LINEAR

30 (ii)MOLECULE TYPE:
 (vi)ORIGINAL SOURCE:
 (A) ORGANISM:

35 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 90:

Glu Trp Gln Thr Asp Asn Cys Glu Thr Cys Thr Cys Tyr Glu Thr Glu
 1 5 10 15

40 Trp Gln Thr Asp Asn Cys Glu Thr Cys Thr Cys Tyr Glu Thr
 20 25 30

45

2) INFORMATION FOR SEQ ID NO: 91:
 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 45
 (B) TYPE: AMINO ACID
 (C) STRANDEDNESS: SINGLE
 (D) TOPOLOGY: LINEAR

50 (ii)MOLECULE TYPE:
 (vi)ORIGINAL SOURCE:
 (A) ORGANISM:

55 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 91:

Glu Trp Gln Thr Asp Asn Cys Glu Thr Cys Thr Cys Tyr Glu Thr Glu
 1 5 10 15

5 Trp Gln Thr Asp Asn Cys Glu Thr Cys Thr Cys Tyr Glu Thr Glu Trp
 20 25 30

Gln Thr Asp Asn Cys Glu Thr Cys Thr Cys Tyr Glu Thr
 35 40 45

10

2) INFORMATION FOR SEQ ID NO: 92:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 60
 (B) TYPE: AMINO ACID
 15 (C) STRANDEDNESS: SINGLE
 (D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE:

(vi) ORIGINAL SOURCE:

20 (A) ORGANISM:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 92:

25 Glu Trp Gln Thr Asp Asn Cys Glu Thr Cys Thr Cys Tyr Glu Thr Glu
 1 5 10 15

Trp Gln Thr Asp Asn Cys Glu Thr Cys Thr Cys Tyr Glu Thr Glu Trp
 20 25 30

30 Gln Thr Asp Asn Cys Glu Thr Cys Thr Cys Tyr Glu Thr Glu Trp Gln
 35 40 45

Thr Asp Asn Cys Glu Thr Cys Thr Cys Tyr Glu Thr
 50 55 60

35

CLAIMS :

1. A polypeptide selected from the group consisting of the polypeptide as set forth in SEQ ID NO: 3, the polypeptide as set forth in SEQ ID NO: 4, the polypeptide as set forth in SEQ ID NO: 5, and the polypeptide as set forth in SEQ ID NO: 6.
2. A polypeptide analog selected from the group consisting of a polypeptide analog of at least five contiguous amino acids of SEQ ID NO: 2, of SEQ ID NO: 3, of SEQ ID NO: 4, of SEQ ID NO: 5, or of SEQ ID NO: 6, a polypeptide analog of at least two contiguous amino acids of SEQ ID NO: 2, of SEQ ID NO: 3, of SEQ ID NO: 4, of SEQ ID NO: 5, or of SEQ ID NO: 6, a polypeptide analog consisting of the amino acid sequence $X_1 W Q X_2 D X_1 C X_1 X_2 C X_2 C X_3 X_1 X_2$ as set forth in SEQ ID NO: 89, wherein X_1 is either glutamic acid (Glu), asparagine (Asn) or aspartic acid (Asp), X_2 is either threonine (Thr) or serine (Ser), and X_3 is either tyrosine (Tyr) or phenylalanine (Phe), a polypeptide analog comprising SEQ ID NO: 5 and having an addition of at least one amino acid to its amino-terminus, wherein said polypeptide analog comprising SEQ ID NO: 5 is selected from the group consisting of SEQ ID NO: 59 to SEQ ID NO: 88, a polypeptide analog comprising SEQ ID NO: 5 and having an addition of at least one amino acid to its carboxy-terminus, wherein said polypeptide analog comprising SEQ ID NO: 5 is selected from the group consisting of SEQ ID NO: 10 to SEQ ID NO: 58, a polypeptide analog comprising two to fifty units of SEQ ID NO: 5, a polypeptide analog comprising two to ten units of SEQ ID NO: 5, a polypeptide analog consisting of a sequence of from two to fourteen amino acid units wherein the amino acid units are selected from the group of amino acid units of SEQ ID NO: 5 consisting of glutamic acid (Glu), tryptophan (Trp), glutamine (Gln), threonine (Thr), aspartic acid (Asp), asparagine (Asn), cysteine (Cys), or tyrosine (Tyr), a polypeptide analog having at least 90 % of its amino acid sequence identical to the amino acid sequence set forth in SEQ ID NO: 5, a polypeptide analog having at least 70 % of its amino acid sequence identical to the amino acid sequence set forth in SEQ ID NO: 5, and a polypeptide analog having at least 50 % of its amino acid sequence identical to the amino acid sequence set forth in SEQ ID NO: 5, said polypeptide analog being capable of inhibiting the growth of prostatic adenocarcinoma, stomach cancer, breast cancer, endometrial,

ovarian or other cancers of epithelial secretion, or benign prostate hyperplasia (BPH).

3. A polypeptide analog selected from the group consisting of a polypeptide analog of at least five contiguous amino acids of SEQ ID NO: 2, of SEQ ID NO: 3, of SEQ ID NO: 4, of SEQ ID NO: 5, or of SEQ ID NO: 6, a polypeptide analog of at least two contiguous amino acids of SEQ ID NO: 2, of SEQ ID NO: 3, of SEQ ID NO: 4, of SEQ ID NO: 5, or of SEQ ID NO: 6, a polypeptide analog consisting of the amino acid sequence $X_1 W Q X_2 D X_1 C X_1 X_2 C X_2 C X_3 X_1 X_2$ as set forth in SEQ ID NO: 89, wherein X_1 is either glutamic acid (Glu), asparagine (Asn) or aspartic acid (Asp), X_2 is either threonine (Thr) or serine (Ser), and X_3 is either tyrosine (Tyr) or phenylalanine (Phe), a polypeptide analog comprising SEQ ID NO: 5 and having an addition of at least one amino acid to its amino-terminus, wherein said polypeptide analog comprising SEQ ID NO: 5 is selected from the group consisting of SEQ ID NO: 59 to SEQ ID NO: 88, a polypeptide analog comprising SEQ ID NO: 5 and having an addition of at least one amino acid to its carboxy-terminus, wherein said polypeptide analog comprising SEQ ID NO: 5 is selected from the group consisting of SEQ ID NO: 10 to SEQ ID NO: 58, a polypeptide analog comprising two to fifty units of SEQ ID NO: 5, a polypeptide analog comprising two to ten units of SEQ ID NO: 5, a polypeptide analog consisting of a sequence of from two to fourteen amino acid units wherein the amino acid units are selected from the group of amino acid units of SEQ ID NO: 5 consisting of glutamic acid (Glu), tryptophan (Trp), glutamine (Gln), threonine (Thr), aspartic acid (Asp), asparagine (Asn), cysteine (Cys), or tyrosine (Tyr), a polypeptide analog having at least 90 % of its amino acid sequence identical to the amino acid sequence set forth in SEQ ID NO: 5, a polypeptide analog having at least 70 % of its amino acid sequence identical to the amino acid sequence set forth in SEQ ID NO: 5, and a polypeptide analog having at least 50 % of its amino acid sequence identical to the amino acid sequence set forth in SEQ ID NO: 5, said polypeptide analog being capable of inhibiting the growth of a tumor.
4. The use of a polypeptide selected from the group consisting of rHuPSP94 as set forth in SEQ ID NO: 2, the decapeptide as set forth in SEQ ID NO: 3, the polypeptide as set forth in SEQ ID NO: 4 (polypeptide 7-21), the polypeptide as set forth in SEQ ID NO: 5 (PCK3145), and the polypeptide as set forth in SEQ ID

NO: 6 (polypeptide 76-94) and mixture(s) thereof, for inhibiting growth of prostatic adenocarcinoma, stomach cancer, breast cancer, endometrial, ovarian or other cancers of epithelial secretion, or benign prostate hyperplasia (BPH).

5

5. The use of a polypeptide selected from the group consisting of rHuPSP94 as set forth in SEQ ID NO: 2, the decapeptide as set forth in SEQ ID NO: 3, the polypeptide as set forth in SEQ ID NO: 4 (polypeptide 7-21), the polypeptide as set forth in SEQ ID NO: 5 (PCK3145), and the polypeptide as set forth in SEQ ID NO: 6 (polypeptide 76-94) and mixture(s) thereof, for inhibiting the growth of a tumor.

10

6. The use of rHuPSP94 as set forth in SEQ ID NO: 2 according to claim 4 or 5 wherein rHuPSP94 is used in a dosage range from about 10 micrograms/kg/day to about 4 milligrams/kg/day.

15

7. The use of rHuPSP94 as set forth in SEQ ID NO: 2 according to claim 4 or 5 wherein rHuPSP94 is used in a dosage range from about 500 picograms/kg/day to about 1 milligram/kg/day.

20

8. The use of rHuPSP94 as set forth in SEQ ID NO: 2 according to claim 4 or 5 wherein rHuPSP94 is used in a dosage range from about 5 nanograms/kg/day to about 10 micrograms/kg/day.

25

9. The use of rHuPSP94 as set forth in SEQ ID NO: 2 according to claim 4 or 5 wherein rHuPSP94 is used in a dosage range from about 5 nanograms/kg/day to about 500 nanograms/kg/day.

30

10. The use of a polypeptide according to claim 4 or 5 wherein said polypeptide is selected from the group consisting of the decapeptide as set forth in SEQ ID NO: 3, the polypeptide as set forth in SEQ ID NO: 4, the polypeptide as set forth in SEQ ID NO: 5, the polypeptide as set forth in SEQ ID NO: 6, and mixtures thereof wherein said polypeptide is used in a dosage range from about 100 nanograms/kg/day to about 4 milligrams/kg/day.

35

11. The use of a polypeptide according to claim 4 or 5 wherein said polypeptide is used with an anticancer drug

40

12. The use of a polypeptide according to claim 11 wherein said anticancer drug is selected from the group consisting of mitomycin, idarubicin, cisplatin, 5-fluoro-uracil,

methotrexate, adriamycin, daunomycin, taxol, taxol derivative, and mixtures thereof.

- 5 13. The use of a polypeptide as in one of claims 4 or 5 wherein said polypeptide is used with a pharmaceutically acceptable carrier.
- 10 14. The use of a polypeptide according to claim 11 wherein said polypeptide is used with a pharmaceutically acceptable carrier.
- 15 15. The use of a polypeptide as in one of claims 4 or 5 wherein said polypeptide is used with a time-release means selected from the group consisting of liposomes and polysaccharides for effecting continual dosing of said polypeptide.
- 20 16. The use of a polypeptide according to claim 11 wherein said polypeptide is used with a time-release means selected from the group consisting of liposomes and polysaccharides for effecting continual dosing of said polypeptide.
- 25 17. The use of a polypeptide according to claim 13 wherein said polypeptide is used with a time-release means selected from the group consisting of liposomes and polysaccharides for effecting continual dosing of said polypeptide.
- 30 18. The use of a polypeptide according to claim 14 wherein said polypeptide is used with a time-release means selected from the group consisting of liposomes and polysaccharides for effecting continual dosing of said polypeptide.
- 35 19. The use of a polypeptide analog selected from the group consisting of a polypeptide analog of at least five contiguous amino acids of SEQ ID NO: 2, of SEQ ID NO: 3, of SEQ ID NO: 4, of SEQ ID NO: 5, or of SEQ ID NO: 6, a polypeptide analog of at least two contiguous amino acids of SEQ ID NO: 2, of SEQ ID NO: 3, of SEQ ID NO: 4, of SEQ ID NO: 5, or of SEQ ID NO: 6, a polypeptide analog consisting of the amino acid sequence $X_1 W Q X_2 D X_1 C X_1 X_2 C X_2 C X_3 X_1 X_2$ as set forth in SEQ ID NO: 89, wherein X_1 is either glutamic acid (Glu), asparagine (Asn) or aspartic acid (Asp), X_2 is either threonine (Thr) or serine (Ser), and X_3 is either tyrosine (Tyr) or phenylalanine (Phe), a polypeptide analog comprising SEQ ID NO: 5 and having an addition of at least one amino acid to its amino-terminus, wherein said polypeptide analog comprising SEQ ID NO: 5 is
- 40

- selected from the group consisting of SEQ ID NO: 59 to SEQ ID NO: 88, a polypeptide analog comprising SEQ ID NO: 5 and having an addition of at least one amino acid to its carboxy-terminus, wherein said polypeptide analog comprising SEQ ID NO:5 is
- 5 selected from the group consisting of SEQ ID NO: 10 to SEQ ID NO: 58, a polypeptide analog comprising two to fifty units of SEQ ID NO: 5, a polypeptide analog comprising two to ten units of SEQ ID NO: 5, a polypeptide analog consisting of a sequence of from two to fourteen amino acid units wherein the amino acid
- 10 units are selected from the group of amino acid units of SEQ ID NO: 5 consisting of glutamic acid (Glu), tryptophan (Trp), glutamine (Gln), threonine (Thr), aspartic acid (Asp), asparagine (Asn), cysteine (Cys), or tyrosine (Tyr), a polypeptide analog having at least 90 % of its amino acid
- 15 sequence identical to the amino acid sequence set forth in SEQ ID NO: 5, a polypeptide analog having at least 70 % of its amino acid sequence identical to the amino acid sequence set forth in SEQ ID NO: 5, and a polypeptide analog having at least 50 % of its amino acid sequence identical to the amino acid
- 20 sequence set forth in SEQ ID NO: 5 for inhibiting growth of prostatic adenocarcinoma, stomach cancer, breast cancer, endometrial, ovarian or other cancers of epithelial secretion, or benign prostate hyperplasia (BPH).
- 25 20. The use of a polypeptide analog selected from the group consisting of a polypeptide analog of at least five contiguous amino acids of SEQ ID NO: 2, of SEQ ID NO: 3, of SEQ ID NO: 4, of SEQ ID NO: 5, or of SEQ ID NO: 6, a polypeptide analog of at least two contiguous amino acids of SEQ ID NO: 2, of SEQ ID NO:
- 30 3, of SEQ ID NO: 4, of SEQ ID NO: 5, or of SEQ ID NO: 6, a polypeptide analog consisting of the amino acid sequence $X_1 W Q X_2 D X_1 C X_1 X_2 C X_2 C X_3 X_1 X_2$ as set forth in SEQ ID NO: 89, wherein X_1 is either glutamic acid (Glu), asparagine (Asn) or aspartic acid (Asp), X_2 is either threonine (Thr) or serine
- 35 (Ser), and X_3 is either tyrosine (Tyr) or phenylalanine (Phe), a polypeptide analog comprising SEQ ID NO: 5 and having an addition of at least one amino acid to its amino-terminus, wherein said polypeptide analog comprising SEQ ID NO:5 is selected from the group consisting of SEQ ID NO: 59 to SEQ ID
- 40 NO: 88, a polypeptide analog comprising SEQ ID NO: 5 and having an addition of at least one amino acid to its carboxy-terminus, wherein said polypeptide analog comprising SEQ ID NO:5 is selected from the group consisting of SEQ ID NO: 10 to SEQ ID NO: 58, a polypeptide analog comprising two to fifty units of

SEQ ID NO: 5, a polypeptide analog comprising two to ten units of SEQ ID NO: 5, a polypeptide analog consisting of a sequence of from two to fourteen amino acid units wherein the amino acid units are selected from the group of amino acid units of SEQ ID NO: 5 consisting of glutamic acid (Glu), tryptophan (Trp), glutamine (Gln), threonine (Thr), aspartic acid (Asp), asparagine (Asn), cysteine (Cys), or tyrosine (Tyr), a polypeptide analog having at least 90 % of its amino acid sequence identical to the amino acid sequence set forth in SEQ ID NO: 5, a polypeptide analog having at least 70 % of its amino acid sequence identical to the amino acid sequence set forth in SEQ ID NO: 5, and a polypeptide analog having at least 50 % of its amino acid sequence identical to the amino acid sequence set forth in SEQ ID NO: 5 for inhibiting the growth of a tumor.

21. The use of a polypeptide analog according to claim 19 or 20 wherein said polypeptide analog is used with an anticancer drug.

22. The use of a polypeptide analog according to claim 21, wherein said anticancer drug is selected from the group consisting of mitomycin, idarubicin, cisplatin, 5-fluoro-uracil, methotrexate, adriamycin, daunomycin, taxol, taxol derivative, and mixtures thereof.

23. The use of a polypeptide analog according to claim 19 or 20 wherein said polypeptide analog is used with a pharmaceutically acceptable carrier.

24. The use of a polypeptide analog according to claim 21 wherein said polypeptide analog is used with a pharmaceutically acceptable carrier.

25. The use of a polypeptide analog according to claim 19 or 20, wherein said polypeptide analog is used with a time-release means selected from the group consisting of liposomes and polysaccharides for effecting continual dosing of said polypeptide analog.

26. The use of a polypeptide analog according to claim 21 wherein said polypeptide analog is used with a time-release means selected from the group consisting of liposomes and

polysaccharides for effecting continual dosing of said polypeptide analog.

5 27. The use of a polypeptide analog according to claim 23 wherein said polypeptide analog is used with a time-release means selected from the group consisting of liposomes and polysaccharides for effecting continual dosing of said polypeptide analog.

10 28. The use of a polypeptide analog according to claim 24 wherein said polypeptide analog is used with a time-release means selected from the group consisting of liposomes and polysaccharides for effecting continual dosing of said polypeptide analog.

15 29. A method for treating a patient with prostatic adenocarcinoma, stomach cancer, breast cancer, endometrial, ovarian or other cancers of epithelial secretion, or benign prostate hyperplasia (BPH), the method comprising administering to the patient a
20 pharmaceutical composition comprising a polypeptide selected from the group consisting of rHuPSP94 as set forth in SEQ ID NO: 2, the decapeptide as set forth in SEQ ID NO: 3, the polypeptide as set forth in SEQ ID NO: 4 (polypeptide 7-21), the polypeptide as set forth in SEQ ID NO: 5 (PCK3145), and the
25 polypeptide as set forth in SEQ ID NO: 6 (polypeptide 76-94) and mixtures thereof.

30 30. A method for treating a patient with a tumor, the method comprising administering to the patient a pharmaceutical composition comprising a polypeptide selected from the group consisting of rHuPSP94 as set forth in SEQ ID NO: 2, the decapeptide as set forth in SEQ ID NO: 3, the polypeptide as set forth in SEQ ID NO: 4 (polypeptide 7-21), the polypeptide as set forth in SEQ ID NO: 5 (PCK3145), and the polypeptide as
35 set forth in SEQ ID NO: 6 (polypeptide 76-94) and mixtures thereof.

40 31. The method according to claim 29 or 30 wherein rHuPSP94 (SEQ ID NO: 2) is administered in a dosage range from about 10 micrograms/kg/day to about 4 milligrams/kg/day.

32. The method according to claim 29 or 30 wherein rHuPSP94 (SEQ ID NO: 2) is administered in a dosage range from about 25 picograms/kg/day to about 1 milligram/kg/day.

33. The method according to claim 29 or 30 wherein human rHuPSP94 (SEQ ID NO: 2) is administered in a dosage range from about 5 nanograms/kg/day to about 10 micrograms/kg/day.
- 5
34. The method according to claim 29 or 30 wherein said polypeptide is selected from the group consisting of the decapeptide as set forth in SEQ ID NO: 3, the polypeptide as set forth in SEQ ID NO: 4, the polypeptide as set forth in SEQ ID NO: 5, the polypeptide as set forth in SEQ ID NO: 6, and mixtures thereof, wherein said polypeptide is used in a dosage range from about 100 nanograms/kg/day to about 4 milligrams/kg/day.
- 10
35. The method according to claim 29 or 30 wherein said polypeptide is used with an anticancer drug.
- 15
36. The method of claim 35 wherein said anticancer drug is selected from the group consisting of mitomycin, idarubicin, cisplatin, 5-fluoro-uracil, methotrexate, adriamycin, daunomycin, taxol, taxol derivative, and mixtures thereof.
- 20
37. The method according to claim 29 or 30 wherein said polypeptide is used with a pharmaceutically acceptable carrier.
- 25
38. The method according to claim 35 wherein said polypeptide is used with a pharmaceutically acceptable carrier.
- 30
39. The method according to claim 29 or 30 wherein said polypeptide is used with a time-release means selected from the group consisting of liposomes and polysaccharides for effecting continual dosing of said polypeptide.
- 35
40. The method according to claim 35 wherein said polypeptide is used with a time-release means selected from the group consisting of liposomes and polysaccharides for effecting continual dosing of said polypeptide.
- 40
41. The method according to claim 37 wherein said polypeptide is used with a time-release means selected from the group consisting of liposomes and polysaccharides for effecting continual dosing of said polypeptide.
42. The method according to claim 38 wherein said polypeptide is used with a time-release means selected from the group

consisting of liposomes and polysaccharides for effecting continual dosing of said polypeptide.

5 43. A method for treating a patient with prostatic adenocarcinoma, stomach cancer, breast cancer, endometrial, ovarian or other cancers of epithelial secretion, or benign prostate hyperplasia (BPH), the method comprising administering to the patient a pharmaceutical composition including a vector comprising the nucleotide sequence of SEQ ID NO: 9 and a pharmaceutically acceptable carrier.

10 44. A method for treating a patient with a tumor, the method comprising administering to the patient a pharmaceutical composition including a vector comprising the nucleotide sequence of SEQ ID NO: 9 and a pharmaceutically acceptable carrier.

15 45. The method according to claim 43 or 44 wherein said vector is used with an anticancer drug.

20 46. The method according to claim 45, wherein said anticancer drug is selected from the group consisting of mitomycin, idarubicin, cisplatin, 5-fluoro-uracil, methotrexate, adriamycin, daunomycin, taxol, taxol derivative, and mixtures thereof.

25 47. The method according to claim 43 or 44 wherein said vector is used with a time-release means selected from the group consisting of liposomes and polysaccharides for effecting continual dosing of said vector.

30 48. The method according to claim 45 wherein said vector is used with a time-release means selected from the group consisting of liposomes and polysaccharides for effecting continual dosing of said vector.

35 49. A method for treating a patient with prostatic adenocarcinoma, stomach cancer, breast cancer, endometrial, ovarian or other cancers of epithelial secretion, or benign prostate hyperplasia (BPH), the method comprising administering to the patient a pharmaceutical composition comprising a polynucleotide selected from the group consisting of a polynucleotide having at least 10 to 285 contiguous residues of SEQ ID NO: 9, and a polynucleotide having at least 10 to 50 contiguous residues of SEQ ID NO: 9, and a pharmaceutically acceptable carrier.

50. A method for treating a patient with a tumor, the method comprising administering to the patient a pharmaceutical composition comprising a polynucleotide selected from the group consisting of a polynucleotide having at least 10 to 285 contiguous residues of SEQ ID NO: 9, and a polynucleotide having at least 10 to 50 contiguous residues of SEQ ID NO: 9, and a pharmaceutically acceptable carrier.

51. The method according to claim 49 or 50 wherein said polynucleotide is used with an anticancer drug.

52. The method according to claim 51, wherein said anticancer drug is selected from the group consisting of mitomycin, idarubicin, cisplatin, 5-fluoro-uracil, methotrexate, adriamycin, daunomycin, taxol, taxol derivative, and mixtures thereof.

53. The method according to claim 49 or 50 wherein said polynucleotide is used with a time-release means selected from the group consisting of liposomes and polysaccharides for effecting continual dosing of said polynucleotide.

54. The method according to claim 51 wherein said polynucleotide is used with a time-release means selected from the group consisting of liposomes and polysaccharides for effecting continual dosing of said polynucleotide.

55. A method for treating a patient with prostatic adenocarcinoma, stomach cancer, breast cancer, endometrial, ovarian or other cancers of epithelial secretion, or benign prostate hyperplasia (BPH), the method comprising administering to the patient a pharmaceutical composition comprising a polypeptide analog selected from the group consisting of a polypeptide analog of at least five contiguous amino acids of SEQ ID NO: 2, of SEQ ID NO: 3, of SEQ ID NO: 4, of SEQ ID NO: 5, or of SEQ ID NO: 6, a polypeptide analog of at least two contiguous amino acids of SEQ ID NO: 2, of SEQ ID NO: 3, of SEQ ID NO: 4, of SEQ ID NO: 5, or of SEQ ID NO: 6, a polypeptide analog consisting of the amino acid sequence $X_1 W Q X_2 D X_1 C X_1 X_2 C X_2 C X_3 X_1 X_2$ as set forth in SEQ ID NO: 89, wherein X_1 is either glutamic acid (Glu), asparagine (Asn) or aspartic acid (Asp), X_2 is either threonine (Thr) or serine (Ser), and X_3 is either tyrosine (Tyr) or phenylalanine (Phe), a polypeptide analog comprising SEQ ID NO: 5 and having an addition of at least one amino acid

to its amino-terminus, wherein said polypeptide analog comprising SEQ ID NO:5 is selected from the group consisting of SEQ ID NO: 59 to SEQ ID NO: 88, a polypeptide analog comprising SEQ ID NO: 5 and having an addition of at least one amino acid to its carboxy-terminus, wherein said polypeptide analog comprising SEQ ID NO:5 is selected from the group consisting of SEQ ID NO: 10 to SEQ ID NO: 58, a polypeptide analog comprising two to fifty units of SEQ ID NO: 5, a polypeptide analog comprising two to ten units of SEQ ID NO: 5, a polypeptide analog consisting of a sequence of from two to fourteen amino acid units wherein the amino acid units are selected from the group of amino acid units of SEQ ID NO: 5 consisting of glutamic acid (Glu), tryptophan (Trp), glutamine (Gln), threonine (Thr), aspartic acid (Asp), asparagine (Asn), cysteine (Cys), or tyrosine (Tyr), a polypeptide analog having at least 90 % of its amino acid sequence identical to the amino acid sequence set forth in SEQ ID NO: 5, a polypeptide analog having at least 70 % of its amino acid sequence identical to the amino acid sequence set forth in SEQ ID NO: 5, and a polypeptide analog having at least 50 % of its amino acid sequence identical to the amino acid sequence set forth in SEQ ID NO: 5, said polypeptide analog being capable of inhibiting the growth of prostatic adenocarcinoma, stomach cancer, breast cancer, endometrial, ovarian or other cancers of epithelial secretion, or benign prostate hyperplasia (BPH).

56.A method for treating a patient with a tumor, the method comprising administering to the patient a pharmaceutical composition comprising a polypeptide analog selected from the group consisting of a polypeptide analog of at least five contiguous amino acids of SEQ ID NO: 2, of SEQ ID NO: 3, of SEQ ID NO: 4, of SEQ ID NO: 5, or of SEQ ID NO: 6, a polypeptide analog of at least two contiguous amino acids of SEQ ID NO: 2, of SEQ ID NO: 3, of SEQ ID NO: 4, of SEQ ID NO: 5, or of SEQ ID NO: 6, a polypeptide analog consisting of the amino acid sequence $X_1 W Q X_2 D X_1 C X_1 X_2 C X_2 C X_3 X_1 X_2$ as set forth in SEQ ID NO: 89, wherein X_1 is either glutamic acid (Glu), asparagine (Asn) or aspartic acid (Asp), X_2 is either threonine (Thr) or serine (Ser), and X_3 is either tyrosine (Tyr) or phenylalanine (Phe), a polypeptide analog comprising SEQ ID NO: 5 and having an addition of at least one amino acid to its amino-terminus, wherein said polypeptide analog comprising SEQ ID NO:5 is selected from the group consisting of SEQ ID NO: 59 to SEQ ID NO: 88, a polypeptide analog comprising SEQ ID NO: 5 and having

an addition of at least one amino acid to its carboxy-terminus, wherein said polypeptide analog comprising SEQ ID NO:5 is selected from the group consisting of SEQ ID NO: 10 to SEQ ID NO: 58, a polypeptide analog comprising two to fifty units of SEQ ID NO: 5, a polypeptide analog comprising two to ten units of SEQ ID NO: 5, a polypeptide analog consisting of a sequence of from two to fourteen amino acid units wherein the amino acid units are selected from the group of amino acid units of SEQ ID NO: 5 consisting of glutamic acid (Glu), tryptophan (Trp), glutamine (Gln), threonine (Thr), aspartic acid (Asp), asparagine (Asn), cysteine (Cys), or tyrosine (Tyr), a polypeptide analog having at least 90 % of its amino acid sequence identical to the amino acid sequence set forth in SEQ ID NO: 5, a polypeptide analog having at least 70 % of its amino acid sequence identical to the amino acid sequence set forth in SEQ ID NO: 5, and a polypeptide analog having at least 50 % of its amino acid sequence identical to the amino acid sequence set forth in SEQ ID NO: 5, said polypeptide analog being capable of inhibiting the growth a tumor.

57.The method according to claim 55 or 56 wherein said polypeptide analog is used with an anticancer drug.

58.The method according to claim 57, wherein said anticancer drug is selected from the group consisting of mitomycin, idarubicin, cisplatin, 5-fluoro-uracil, methotrexate, adriamycin, daunomycin, taxol, taxol derivative, and mixtures thereof.

59.The method according to claim 55 or 56, wherein said polypeptide analog is used with a pharmaceutically acceptable carrier.

60.The method according to claim 57, wherein said polypeptide analog is used with a pharmaceutically acceptable carrier.

61.The method according to claim 55 or 56, wherein said polypeptide analog is used with a time-release means selected from the group consisting of liposomes and polysaccharides for effecting continual dosing of said polypeptide analog.

62.The method according to claim 57 wherein said polypeptide analog is used with a time-release means selected from the group consisting of liposomes and polysaccharides for effecting continual dosing of said polypeptide analog.

63. The method according to claim 59 wherein said polypeptide analog is used with a time-release means selected from the group consisting of liposomes and polysaccharides for effecting continual dosing of said polypeptide analog.

64. The method according to claim 60 wherein said polypeptide analog is used with a time-release means selected from the group consisting of liposomes and polysaccharides for effecting continual dosing of said polypeptide analog.

65. A pharmaceutical composition for inhibiting the growth of a tumor in a patient suffering from prostatic adenocarcinoma, stomach cancer, breast cancer, endometrial, ovarian or other cancers of epithelial secretion, or benign prostate hyperplasia (BPH), comprising:

a) a polypeptide selected from the group consisting of rHuPSP94 as set forth in SEQ ID NO: 2, the decapeptide as set forth in SEQ ID NO: 3, the polypeptide as set forth in SEQ ID NO: 4 (Polypeptide 7-21), the polypeptide as set forth in SEQ ID NO: 5 (PCK3145), the polypeptide as set forth in SEQ ID NO: 6 (Polypeptide 76-94) and mixture(s) thereof, and;

b) an anticancer drug.

66. A pharmaceutical composition for inhibiting the growth of a tumor in a patient suffering from prostatic adenocarcinoma, stomach cancer, breast cancer, endometrial, ovarian or other cancers of epithelial secretion, or benign prostate hyperplasia (BPH), comprising:

a) a polypeptide selected from the group consisting of rHuPSP94 as set forth in SEQ ID NO: 2, the decapeptide as set forth in SEQ ID NO: 3, the polypeptide as set forth in SEQ ID NO: 4 (Polypeptide 7-21), the polypeptide as set forth in SEQ ID NO: 5 (PCK3145), the polypeptide as set forth in SEQ ID NO: 6 (Polypeptide 76-94) and mixture(s) thereof, and;

b) a pharmaceutically acceptable carrier.

67. A pharmaceutical composition comprising:

a) A polypeptide selected from the group consisting of rHuPSP94 as set forth in SEQ ID NO: 2, the decapeptide as set forth in SEQ ID NO: 3, the polypeptide as set forth in SEQ ID NO: 4 (polypeptide 7-21), the polypeptide as set forth in SEQ ID NO: 5 (PCK3145), the polypeptide as set forth in SEQ ID NO: 6 (polypeptide 76-94) and mixture(s) thereof in a therapeutically effective amount, and;

b) an anticancer drug in a therapeutically effective amount.

68.A pharmaceutical composition comprising:

a) a polypeptide selected from the group consisting of rHuPSP94 as set forth in SEQ ID NO: 2, the decapeptide as set forth in SEQ ID NO: 3, the polypeptide as set forth in SEQ ID NO: 4 (polypeptide 7-21), the polypeptide as set forth in SEQ ID NO: 5 (PCK3145), the polypeptide as set forth in SEQ ID NO: 6 (polypeptide 76-94) and mixture(s) thereof in a therapeutically effective amount, and;

b) a pharmaceutically acceptable carrier.

69.A pharmaceutical composition as in one claim 65 to 68, wherein rHuPSP94 (SEQ ID NO: 2) is used in a dosage range from about 10 micrograms/kg/day to about 4 milligrams/kg/day.

70.A pharmaceutical composition as in one claim 65 to 68, wherein rHuPSP94 (SEQ ID NO: 2) is used in a dosage range from about 500 picograms/kg/day to about 1 milligram/kg/day.

71.A pharmaceutical composition as in one claim 65 to 68, wherein rHuPSP94 is used in a dosage range from about 5 nanograms/kg/day to about 10 micrograms/kg/day.

72.A pharmaceutical composition as in one claim 65 to 68, wherein rHuPSP94 is used in a dosage range from about 5 nanograms/kg/day to about 500 nanograms/kg/day.

73.A pharmaceutical composition as in one claim 65 to 68, wherein said polypeptide is selected from the group consisting of the decapeptide as set forth in SEQ ID NO: 3, the polypeptide as

set forth in SEQ ID NO: 4, the polypeptide as set forth in SEQ ID NO: 5, the polypeptide as set forth in SEQ ID NO: 6 and mixture(s) thereof, wherein said polypeptide is used in a dosage range from about 100 nanograms/kg/day to about 4 milligrams/kg/day.

74.A pharmaceutical composition according to claim 66 or 68 further comprising an anticancer drug.

75.A pharmaceutical composition according to claim 65, 67 or 74 wherein said anticancer drug is selected from the group consisting of mitomycin, idarubicin, cisplatin, 5-fluorouracil, methotrexate, adriamycin, daunomycin, taxol, taxol derivative, and mixtures thereof.

76.A pharmaceutical composition as in one claim 65 to 68 or 74, further comprising a time-release means selected from the group consisting of liposomes and polysaccharides for effecting continual dosing of the composition.

77.A pharmaceutical composition for inhibiting the growth of a tumor in a patient suffering from prostatic adenocarcinoma, stomach cancer, breast cancer, endometrial, ovarian or other cancers of epithelial secretion, or benign prostate hyperplasia (BPH), comprising a vector comprising the nucleotide sequence of SEQ ID NO: 9 and a pharmaceutically acceptable carrier.

78.A pharmaceutical composition for inhibiting the growth of a tumor in a patient, comprising a vector comprising the nucleotide sequence of SEQ ID NO: 9 and a pharmaceutically acceptable carrier.

79.A pharmaceutical composition for inhibiting the growth of a tumor in a patient suffering from prostatic adenocarcinoma, stomach cancer, breast cancer, endometrial, ovarian or other cancers of epithelial secretion, or benign prostate hyperplasia (BPH), comprising a polynucleotide selected from the group consisting of a polynucleotide having at least 10 to 285 contiguous residues of SEQ ID NO: 9 and a polynucleotide having at least 10 to 50 contiguous residues of SEQ ID NO: 9, and a pharmaceutically acceptable carrier.

80.A pharmaceutical composition for inhibiting the growth of a tumor in a patient, comprising a polynucleotide selected from

the group consisting of a polynucleotide having at least 10 to 285 contiguous residues of SEQ ID NO: 9 and a polynucleotide having at least 10 to 50 contiguous residues of SEQ ID NO: 9, and a pharmaceutically acceptable carrier.

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81.A pharmaceutical composition as in one of claims 77-80 further comprising an anticancer drug.

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82.A pharmaceutical composition according to claim 81 wherein said anticancer drug is selected from the group consisting of mitomycin, idarubicin, cisplatin, 5-fluoro-uracil, methotrexate, adriamycin, daunomycin, taxol, taxol derivative, and mixtures thereof.

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83.A pharmaceutical composition for inhibiting the growth of a tumor in a patient suffering from prostatic adenocarcinoma, stomach cancer, breast cancer, endometrial, ovarian or other cancers of epithelial secretion, or benign prostate hyperplasia (BPH), comprising:

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- a) a polypeptide analog selected from the group consisting of a polypeptide analog of at least five contiguous amino acids of SEQ ID NO: 2, of SEQ ID NO: 3, of SEQ ID NO: 4, of SEQ ID NO: 5, or of SEQ ID NO: 6, a polypeptide analog of at least two contiguous amino acids of SEQ ID NO: 2, of SEQ ID NO: 3, of SEQ ID NO: 4, of SEQ ID NO: 5, or of SEQ ID NO: 6, a polypeptide analog consisting of the amino acid sequence $X_1 W Q X_2 D X_1 C X_1 X_2 C X_2 C X_3 X_1 X_2$ as set forth in SEQ ID NO: 89, wherein X_1 is either glutamic acid (Glu), asparagine (Asn) or aspartic acid (Asp), X_2 is either threonine (Thr) or serine (Ser), and X_3 is either tyrosine (Tyr) or phenylalanine (Phe), a polypeptide analog comprising SEQ ID NO: 5 and having an addition of at least one amino acid to its amino-terminus wherein said polypeptide analog comprising SEQ ID NO:5 is selected from the group consisting of SEQ ID NO: 59 to SEQ ID NO: 88, a polypeptide analog comprising SEQ ID NO: 5 and having an addition of at least one amino acid to its carboxy-terminus, wherein said polypeptide analog comprising SEQ ID NO:5 is selected from the group consisting of SEQ ID NO: 10 to SEQ ID NO: 58, a polypeptide analog comprising two to fifty units of SEQ ID NO: 5, a polypeptide analog comprising two to ten units of SEQ ID NO: 5, a polypeptide analog consisting of

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a sequence of from two to fourteen amino acid units wherein the amino acid units are selected from the group of amino acid units of SEQ ID NO: 5 consisting of glutamic acid (Glu), tryptophan (Trp), glutamine (Gln), threonine (Thr), aspartic acid (Asp), asparagine (Asn), cysteine (Cys), or tyrosine (Tyr), a polypeptide analog having at least 90 % of its amino acid sequence identical to the amino acid sequence set forth in SEQ ID NO: 5, a polypeptide analog having at least 70 % of its amino acid sequence identical to the amino acid sequence set forth in SEQ ID NO: 5, and a polypeptide analog having at least 50 % of its amino acid sequence identical to the amino acid sequence set forth in SEQ ID NO: 5, and mixture(s) thereof, and;

b) an anticancer drug.

84.A pharmaceutical composition for inhibiting the growth of a tumor in a patient suffering from prostatic adenocarcinoma, stomach cancer, breast cancer, endometrial, ovarian or other cancers of epithelial secretion, or benign prostate hyperplasia (BPH), comprising:

a) a polypeptide analog selected from the group consisting of a polypeptide analog of at least five contiguous amino acids of SEQ ID NO: 2, of SEQ ID NO: 3, of SEQ ID NO: 4, of SEQ ID NO: 5, or of SEQ ID NO: 6, a polypeptide analog of at least two contiguous amino acids of SEQ ID NO: 2, of SEQ ID NO: 3, of SEQ ID NO: 4, of SEQ ID NO: 5, or of SEQ ID NO: 6, a polypeptide analog consisting of the amino acid sequence $X_1 W Q X_2 D X_1 C X_1 X_2 C X_2 C X_3 X_1 X_2$ as set forth in SEQ ID NO: 89, wherein X_1 is either glutamic acid (Glu), asparagine (Asn) or aspartic acid (Asp), X_2 is either threonine (Thr) or serine (Ser), and X_3 is either tyrosine (Tyr) or phenylalanine (Phe), a polypeptide analog comprising SEQ ID NO: 5 and having an addition of at least one amino acid to its amino-terminus wherein said polypeptide analog comprising SEQ ID NO:5 is selected from the group consisting of SEQ ID NO: 59 to SEQ ID NO: 88, a polypeptide analog comprising SEQ ID NO: 5 and having an addition of at least one amino acid to its carboxy-terminus, wherein said polypeptide analog comprising SEQ ID NO:5 is selected from the group consisting of SEQ ID NO: 10 to SEQ ID NO: 58, a

polypeptide analog comprising two to fifty units of SEQ ID NO: 5, a polypeptide analog comprising two to ten units of SEQ ID NO: 5, a polypeptide analog consisting of a sequence of from two to fourteen amino acid units wherein the amino acid units are selected from the group of amino acid units of SEQ ID NO: 5 consisting of glutamic acid (Glu), tryptophan (Trp), glutamine (Gln), threonine (Thr), aspartic acid (Asp), asparagine (Asn), cysteine (Cys), or tyrosine (Tyr), a polypeptide analog having at least 90 % of its amino acid sequence identical to the amino acid sequence set forth in SEQ ID NO: 5, a polypeptide analog having at least 70 % of its amino acid sequence identical to the amino acid sequence set forth in SEQ ID NO: 5, and a polypeptide analog having at least 50 % of its amino acid sequence identical to the amino acid sequence set forth in SEQ ID NO: 5, and mixture(s) thereof, and;

a) a pharmaceutically acceptable carrier.

85.A pharmaceutical composition comprising:

a) a polypeptide analog selected from the group consisting of a polypeptide analog of at least five contiguous amino acids of SEQ ID NO: 2, of SEQ ID NO: 3, of SEQ ID NO: 4, of SEQ ID NO: 5, or of SEQ ID NO: 6, a polypeptide analog of at least two contiguous amino acids of SEQ ID NO: 2, of SEQ ID NO: 3, of SEQ ID NO: 4, of SEQ ID NO: 5, or of SEQ ID NO: 6, a polypeptide analog consisting of the amino acid sequence X_1 W Q X_2 D X_1 C X_1 X_2 C X_2 C X_3 X_1 X_2 as set forth in SEQ ID NO: 89, wherein X_1 is either glutamic acid (Glu), asparagine (Asn) or aspartic acid (Asp), X_2 is either threonine (Thr) or serine (Ser), and X_3 is either tyrosine (Tyr) or phenylalanine (Phe), a polypeptide analog comprising SEQ ID NO: 5 and having an addition of at least one amino acid to its amino-terminus, wherein said polypeptide analog comprising SEQ ID NO: 5 is selected from the group consisting of SEQ ID NO: 59 to SEQ ID NO: 88, a polypeptide analog comprising SEQ ID NO: 5 and having an addition of at least one amino acid to its carboxy-terminus, wherein said polypeptide analog comprising SEQ ID NO: 5 is selected from the group consisting of SEQ ID NO: 10 to SEQ ID NO: 58, a polypeptide analog comprising two to fifty units of SEQ ID NO: 5, a polypeptide analog comprising two to ten

units of SEQ ID NO: 5, a polypeptide analog consisting of a sequence of from two to fourteen amino acid units wherein the amino acid units are selected from the group of amino acid units of SEQ ID NO: 5 consisting of glutamic acid (Glu), tryptophan (Trp), glutamine (Gln), threonine (Thr), aspartic acid (Asp), asparagine (Asn), cysteine (Cys), or tyrosine (Tyr), a polypeptide analog having at least 90 % of its amino acid sequence identical to the amino acid sequence set forth in SEQ ID NO: 5, a polypeptide analog having at least 70 % of its amino acid sequence identical to the amino acid sequence set forth in SEQ ID NO: 5, and a polypeptide analog having at least 50 % of its amino acid sequence identical to the amino acid sequence set forth in SEQ ID NO: 5 and mixture(s) thereof in a therapeutically effective amount, and;

b) an anticancer drug in a therapeutically effective amount.

86.A pharmaceutical composition comprising:

a) a polypeptide analog selected from the group consisting of a polypeptide analog of at least five contiguous amino acids of SEQ ID NO: 2, of SEQ ID NO: 3, of SEQ ID NO: 4, of SEQ ID NO: 5, or of SEQ ID NO: 6, a polypeptide analog of at least two contiguous amino acids of SEQ ID NO: 2, of SEQ ID NO: 3, of SEQ ID NO: 4, of SEQ ID NO: 5, or of SEQ ID NO: 6, a polypeptide analog consisting of the amino acid sequence $X_1 W Q X_2 D X_1 C X_1 X_2 C X_2 C X_3 X_1 X_2$ as set forth in SEQ ID NO: 89, wherein X_1 is either glutamic acid (Glu), asparagine (Asn) or aspartic acid (Asp), X_2 is either threonine (Thr) or serine (Ser), and X_3 is either tyrosine (Tyr) or phenylalanine (Phe), a polypeptide analog comprising SEQ ID NO: 5 and having an addition of at least one amino acid to its amino-terminus, wherein said polypeptide analog comprising SEQ ID NO: 5 is selected from the group consisting of SEQ ID NO: 59 to SEQ ID NO: 88, a polypeptide analog comprising SEQ ID NO: 5 and having an addition of at least one amino acid to its carboxy-terminus, wherein said polypeptide analog comprising SEQ ID NO: 5 is selected from the group consisting of SEQ ID NO: 10 to SEQ ID NO: 58, a polypeptide analog comprising two to fifty units of SEQ ID NO: 5, a polypeptide analog comprising two to ten units of SEQ ID NO: 5, a polypeptide analog consisting of a sequence of from two to fourteen amino acid units wherein the amino

acid units are selected from the group of amino acid units of SEQ ID NO: 5 consisting of glutamic acid (Glu), tryptophan (Trp), glutamine (Gln), threonine (Thr), aspartic acid (Asp), asparagine (Asn), cysteine (Cys), or tyrosine (Tyr), a polypeptide analog having at least 90 % of its amino acid sequence identical to the amino acid sequence set forth in SEQ ID NO: 5, a polypeptide analog having at least 70 % of its amino acid sequence identical to the amino acid sequence set forth in SEQ ID NO: 5, and a polypeptide analog having at least 50 % of its amino acid sequence identical to the amino acid sequence set forth in SEQ ID NO: 5 and mixture(s) thereof in a therapeutically effective amount, and;

b) a pharmaceutically acceptable carrier.

87. A pharmaceutical composition according to claim 84 or 86, further comprising an anticancer drug.

88. A pharmaceutical composition according to claim 83, 85 or 87 wherein said anticancer drug is selected from the group consisting of mitomycin, idarubicin, cisplatin, 5-fluorouracil, methotrexate, adriamycin, daunomycin, taxol, taxol derivative, and mixtures thereof.

89. A pharmaceutical composition as in one of claim 83 to 86, or 87 further comprising a time-release means selected from the group consisting of liposomes and polysaccharides for effecting continual dosing of the composition.

90. A method for treating patients with a disease characterized by elevated levels of FSH comprising administering a pharmaceutical composition in an appropriate dosage form, the pharmaceutical composition comprising a polypeptide selected from the group consisting of rHuPSP94 as set forth in SEQ ID NO: 2, the decapeptide as set forth in SEQ ID NO: 3, the polypeptide as set forth in SEQ ID NO: 4, the polypeptide as set forth in SEQ ID NO: 5, and the polypeptide as set forth in SEQ ID NO: 6, and a pharmaceutically acceptable carrier.

91. A method for treating patients with a disease characterized by elevated levels of FSH comprising administering a pharmaceutical composition in an appropriate dosage form, the pharmaceutical composition comprising a polypeptide analog

selected from the group consisting of a polypeptide analog of at least five contiguous amino acids of SEQ ID NO: 2, of SEQ ID NO: 3, of SEQ ID NO: 4, of SEQ ID NO: 5, or of SEQ ID NO: 6, a polypeptide analog of at least two contiguous amino acids of SEQ ID NO: 2, of SEQ ID NO: 3, of SEQ ID NO: 4, of SEQ ID NO: 5, or of SEQ ID NO: 6, a polypeptide analog consisting of the amino acid sequence $X_1 W Q X_2 D X_1 C X_1 X_2 C X_2 C X_3 X_1 X_2$ as set forth in SEQ ID NO: 89, wherein X_1 is either glutamic acid (Glu), asparagine (Asn) or aspartic acid (Asp), X_2 is either threonine (Thr) or serine (Ser), and X_3 is either tyrosine (Tyr) or phenylalanine (Phe), a polypeptide analog comprising SEQ ID NO: 5 and having an addition of at least one amino acid to its amino-terminus, wherein said polypeptide analog comprising SEQ ID NO: 5 is selected from the group consisting of SEQ ID NO: 59 to SEQ ID NO: 88, a polypeptide analog comprising SEQ ID NO: 5 and having an addition of at least one amino acid to its carboxy-terminus, wherein said polypeptide analog comprising SEQ ID NO: 5 is selected from the group consisting of SEQ ID NO: 10 to SEQ ID NO: 58, a polypeptide analog comprising two to fifty units of SEQ ID NO: 5, a polypeptide analog comprising two to ten units of SEQ ID NO: 5, a polypeptide analog consisting of a sequence of from two to fourteen amino acid units wherein the amino acid units are selected from the group of amino acid units of SEQ ID NO: 5 consisting of glutamic acid (Glu), tryptophan (Trp), glutamine (Gln), threonine (Thr), aspartic acid (Asp), asparagine (Asn), cysteine (Cys), or tyrosine (Tyr), a polypeptide analog having at least 90 % of its amino acid sequence identical to the amino acid sequence set forth in SEQ ID NO: 5, a polypeptide analog having at least 70 % of its amino acid sequence identical to the amino acid sequence set forth in SEQ ID NO: 5, and a polypeptide analog having at least 50 % of its amino acid sequence identical to the amino acid sequence set forth in SEQ ID NO: 5 and mixture(s) thereof, and a pharmaceutically acceptable carrier, in a human dose.

92. The use of a polypeptide selected from the group consisting of rHuPSP94 as set forth in SEQ ID NO: 2, the decapeptide as set forth in SEQ ID NO: 3, the polypeptide as set forth in SEQ ID NO: 4 (polypeptide 7-21), the polypeptide as set forth in SEQ ID NO: 5 (PCK3145), and the polypeptide as set forth in SEQ ID NO: 6 (polypeptide 76-94) and mixture(s) thereof, for treating patients with a disease characterized by elevated levels of FSH.

93. The use of a polypeptide analog selected from the group consisting of a polypeptide analog of at least five contiguous amino acids of SEQ ID NO: 2, of SEQ ID NO: 3, of SEQ ID NO: 4, of SEQ ID NO: 5, or of SEQ ID NO: 6, a polypeptide analog of at least two contiguous amino acids of SEQ ID NO: 2, of SEQ ID NO: 3, of SEQ ID NO: 4, of SEQ ID NO: 5, or of SEQ ID NO: 6, a polypeptide analog consisting of the amino acid sequence $X_1 W Q X_2 D X_1 C X_1 X_2 C X_2 C X_3 X_1 X_2$ as set forth in SEQ ID NO: 89, wherein X_1 is either glutamic acid (Glu), asparagine (Asn) or aspartic acid (Asp), X_2 is either threonine (Thr) or serine (Ser), and X_3 is either tyrosine (Tyr) or phenylalanine (Phe), a polypeptide analog comprising SEQ ID NO: 5 and having an addition of at least one amino acid to its amino-terminus, wherein said polypeptide analog comprising SEQ ID NO: 5 is selected from the group consisting of SEQ ID NO: 59 to SEQ ID NO: 88, a polypeptide analog comprising SEQ ID NO: 5 and having an addition of at least one amino acid to its carboxy-terminus, wherein said polypeptide analog comprising SEQ ID NO: 5 is selected from the group consisting of SEQ ID NO: 10 to SEQ ID NO: 58, a polypeptide analog comprising two to fifty units of SEQ ID NO: 5, a polypeptide analog comprising two to ten units of SEQ ID NO: 5, a polypeptide analog consisting of a sequence of from two to fourteen amino acid units wherein the amino acid units are selected from the group of amino acid units of SEQ ID NO: 5 consisting of glutamic acid (Glu), tryptophan (Trp), glutamine (Gln), threonine (Thr), aspartic acid (Asp), asparagine (Asn), cysteine (Cys), or tyrosine (Tyr), a polypeptide analog having at least 90 % of its amino acid sequence identical to the amino acid sequence set forth in SEQ ID NO: 5, a polypeptide analog having at least 70 % of its amino acid sequence identical to the amino acid sequence set forth in SEQ ID NO: 5, and a polypeptide analog having at least 50 % of its amino acid sequence identical to the amino acid sequence set forth in SEQ ID NO: 5 and mixture(s) thereof, for treating patients with a disease characterized by elevated levels of FSH.

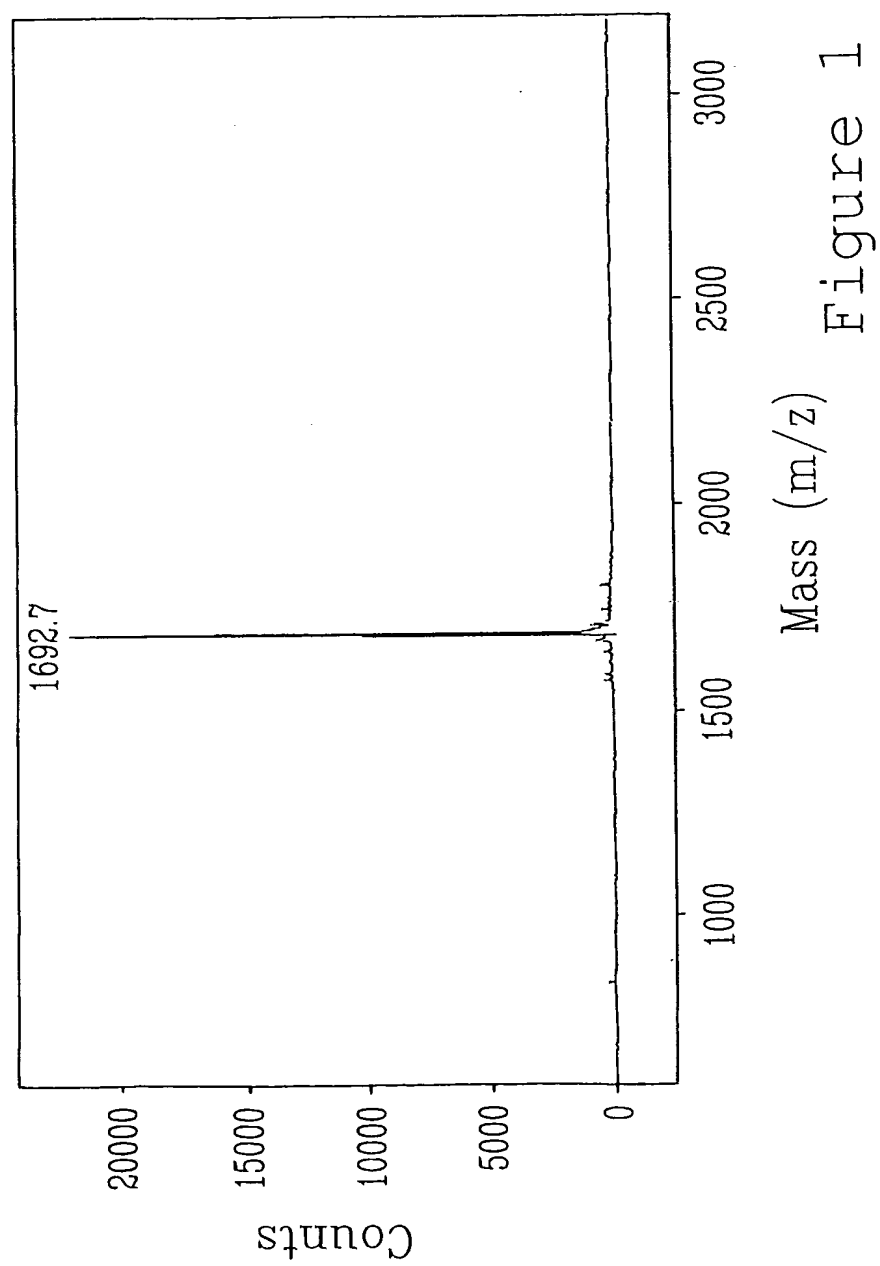
94. The use of a polypeptide selected from the group consisting of rHuPSP94 as set forth in SEQ ID NO: 2, the decapeptide as set forth in SEQ ID NO: 3, the polypeptide as set forth in SEQ ID NO: 4 (polypeptide 7-21), the polypeptide as set forth in SEQ ID NO: 5 (PCK3145), the polypeptide as set forth in SEQ ID NO: 6 (polypeptide 76-94) and mixtures thereof for the manufacture

of a medicament for the therapeutic treatment of prostatic adenocarcinoma, stomach cancer, breast cancer, endometrial, ovarian or other cancers of epithelial secretion, benign prostate hyperplasia (BPH) or a disease characterized by elevated levels of FSH.

95. The use of a polypeptide analog selected from the group consisting of a polypeptide analog of at least five contiguous amino acids of SEQ ID NO: 2, of SEQ ID NO: 3, of SEQ ID NO: 4, of SEQ ID NO: 5, or of SEQ ID NO: 6, a polypeptide analog of at least two contiguous amino acids of SEQ ID NO: 2, of SEQ ID NO: 3, of SEQ ID NO: 4, of SEQ ID NO: 5, or of SEQ ID NO: 6, a polypeptide analog consisting of the amino acid sequence $X_1 W Q X_2 D X_1 C X_1 X_2 C X_2 C X_3 X_1 X_2$ as set forth in SEQ ID NO: 89, wherein X_1 is either glutamic acid (Glu), asparagine (Asn) or aspartic acid (Asp), X_2 is either threonine (Thr) or serine (Ser), and X_3 is either tyrosine (Tyr) or phenylalanine (Phe), a polypeptide analog comprising SEQ ID NO: 5 and having an addition of at least one amino acid to its amino-terminus, wherein said polypeptide analog comprising SEQ ID NO: 5 is selected from the group consisting of SEQ ID NO: 59 to SEQ ID NO: 88, a polypeptide analog comprising SEQ ID NO: 5 and having an addition of at least one amino acid to its carboxy-terminus, wherein said polypeptide analog comprising SEQ ID NO: 5 is selected from the group consisting of SEQ ID NO: 10 to SEQ ID NO: 58, a polypeptide analog comprising two to fifty units of SEQ ID NO: 5, a polypeptide analog comprising two to ten units of SEQ ID NO: 5, a polypeptide analog consisting of a sequence of from two to fourteen amino acid units wherein the amino acid units are selected from the group of amino acid units of SEQ ID NO: 5 consisting of glutamic acid (Glu), tryptophan (Trp), glutamine (Gln), threonine (Thr), aspartic acid (Asp), asparagine (Asn), cysteine (Cys), or tyrosine (Tyr), a polypeptide analog having at least 90 % of its amino acid sequence identical to the amino acid sequence set forth in SEQ ID NO: 5, a polypeptide analog having at least 70 % of its amino acid sequence identical to the amino acid sequence set forth in SEQ ID NO: 5, and a polypeptide analog having at least 50 % of its amino acid sequence identical to the amino acid sequence set forth in SEQ ID NO: 5 and mixture(s) thereof for the manufacture of a medicament for the therapeutic treatment of prostatic adenocarcinoma, stomach cancer, breast cancer, endometrial, ovarian or other cancers of epithelial secretion,

benign prostate hyperplasia (BPH) or a disease characterized by elevated levels of FSH.

1/30



2/30

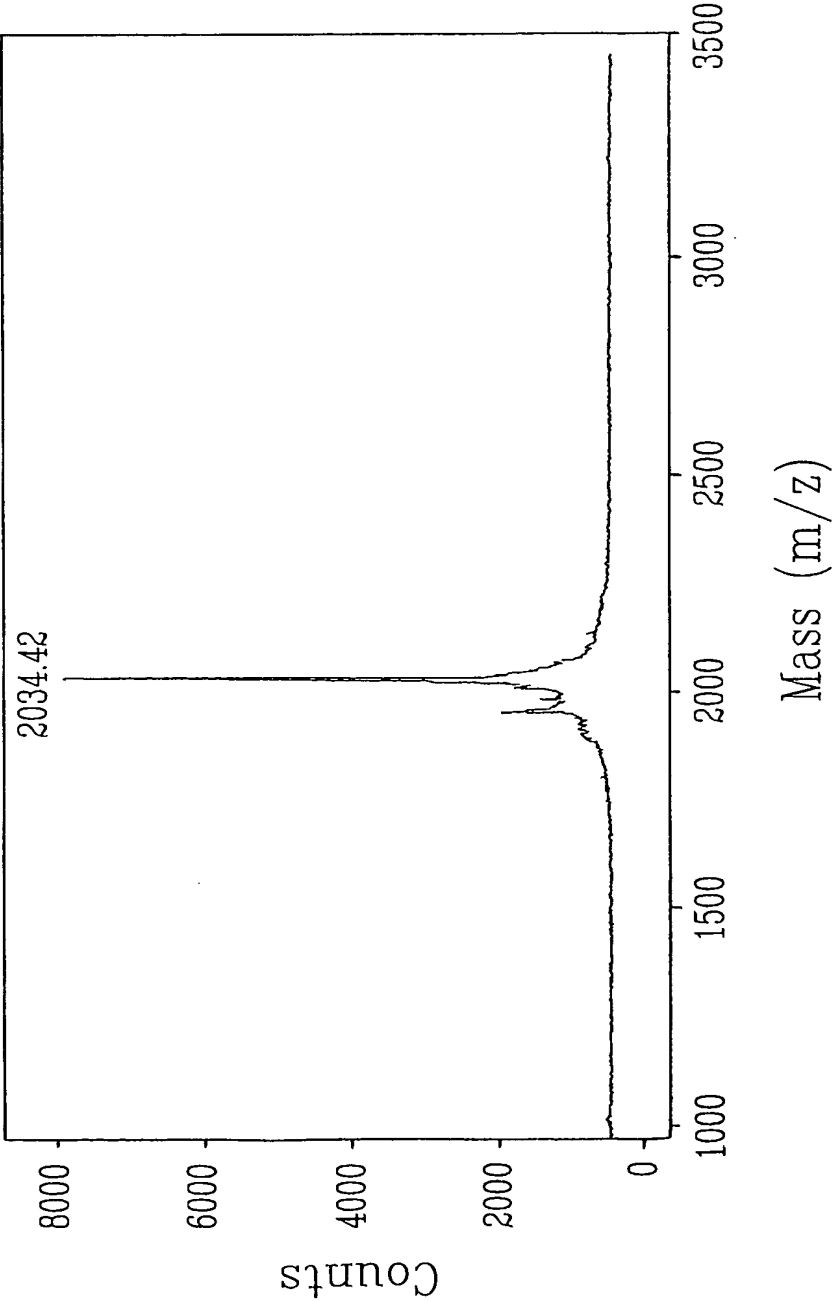
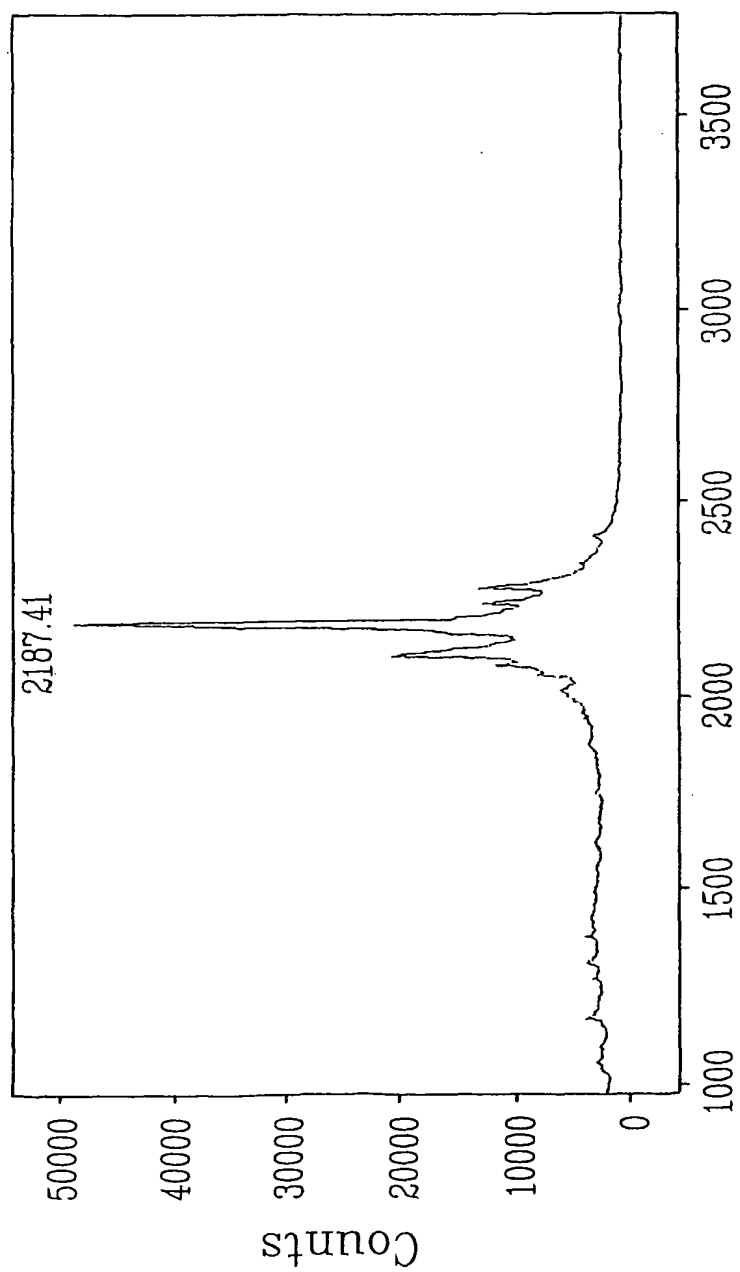


Figure 2

3/30

Mass (m/z) Figure 3

4/30

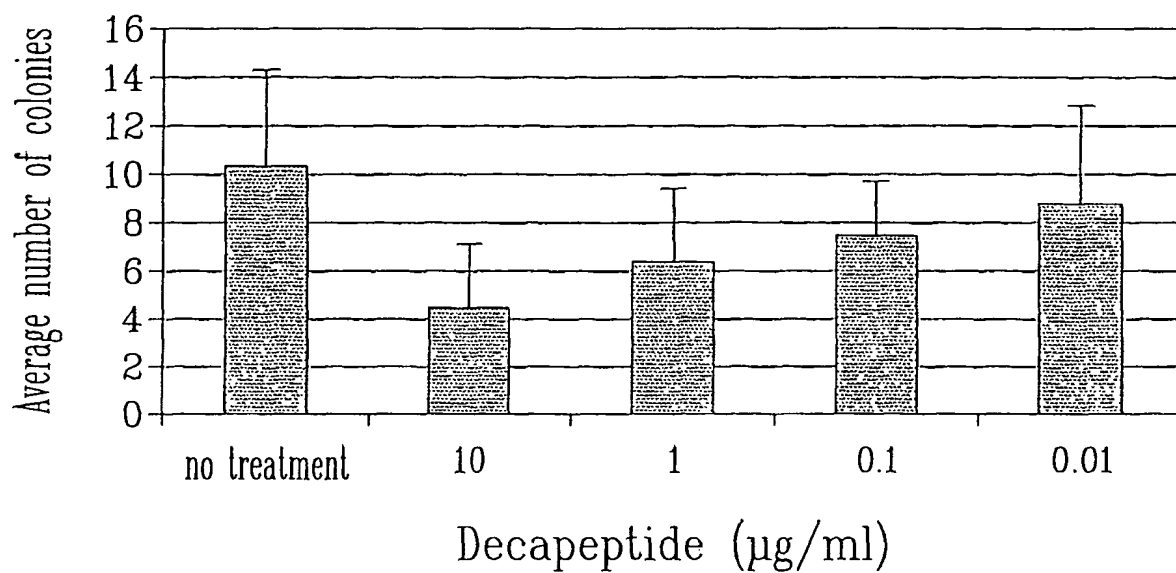


Figure 4a

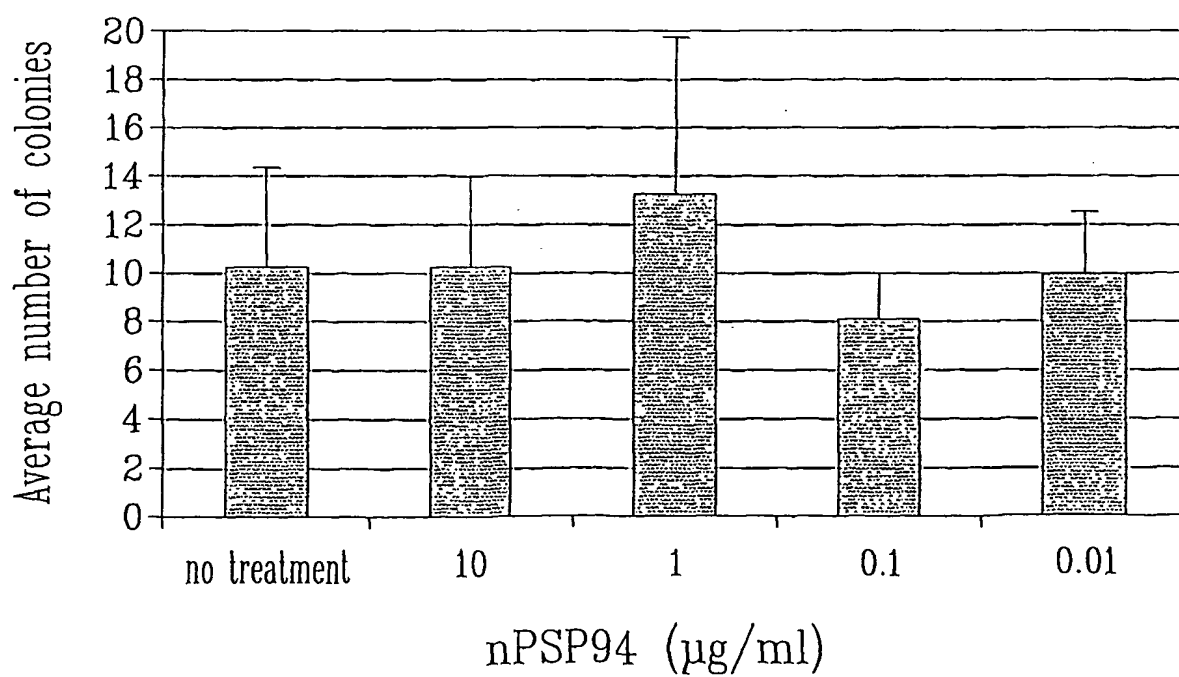


Figure 4b

5/30

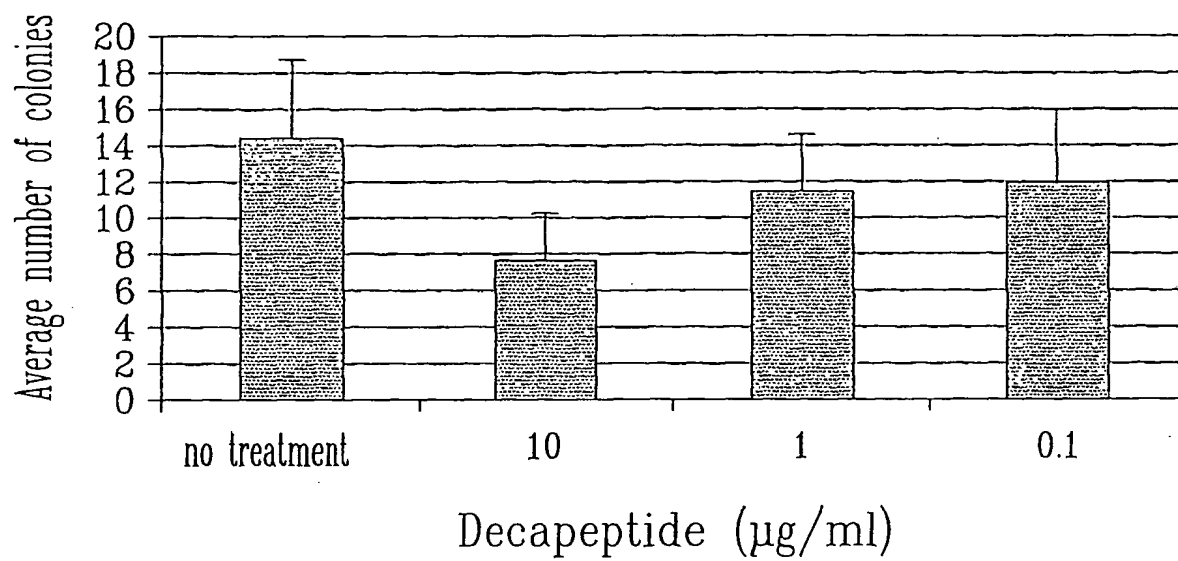


Figure 5a

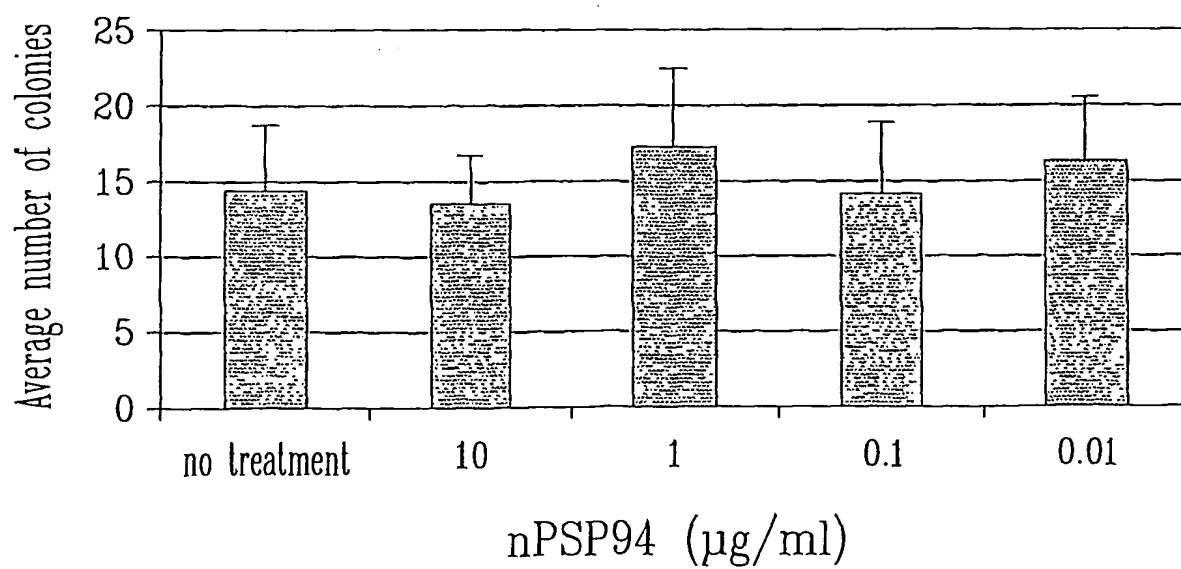


Figure 5b

6/30

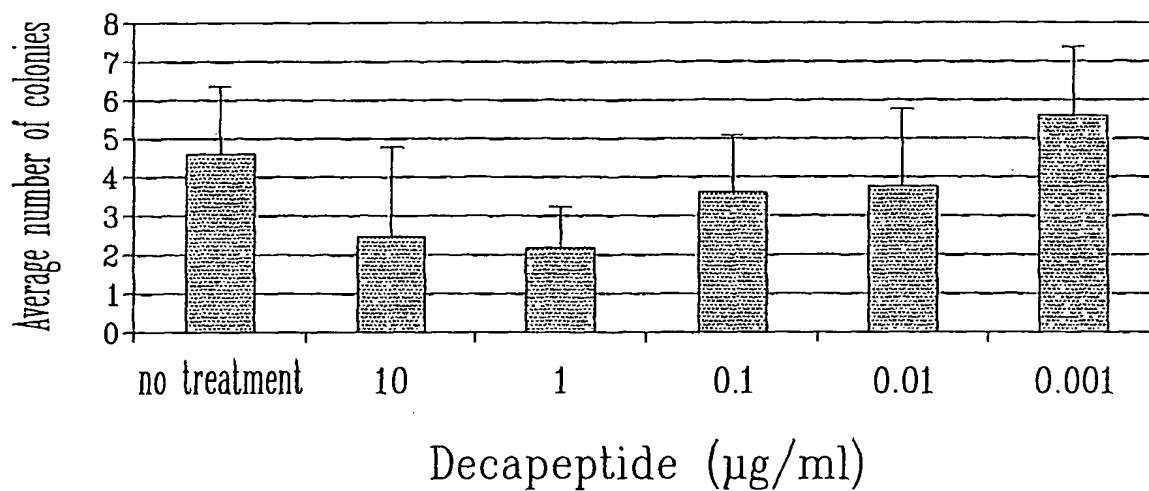


Figure 6a

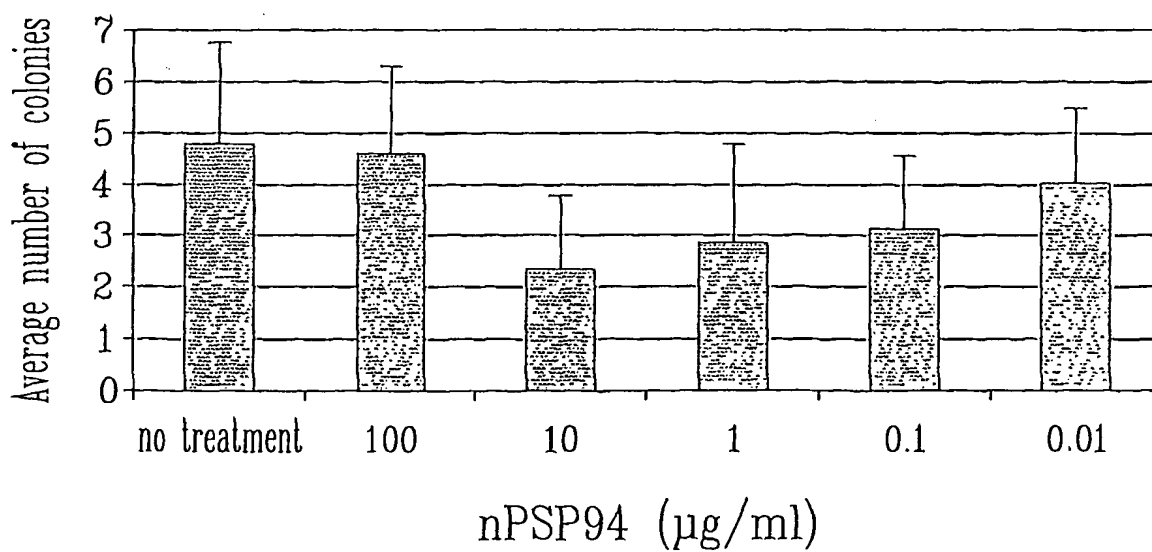


Figure 6b

7/30

1 2 3 4 5 6

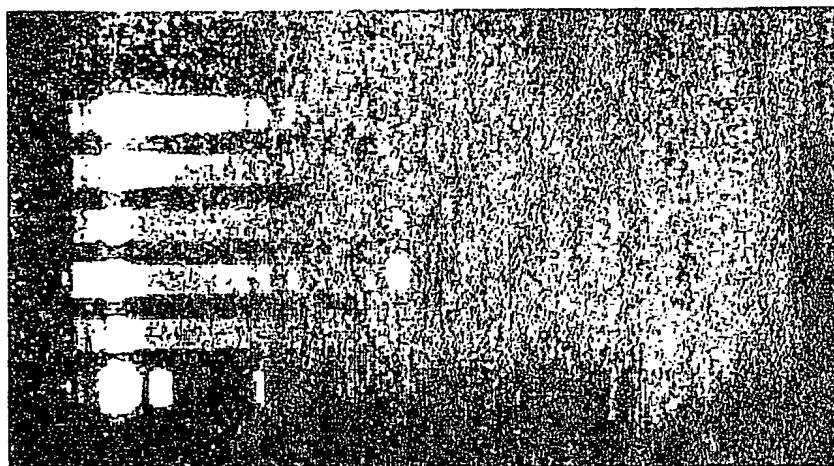


Figure 7

8/30

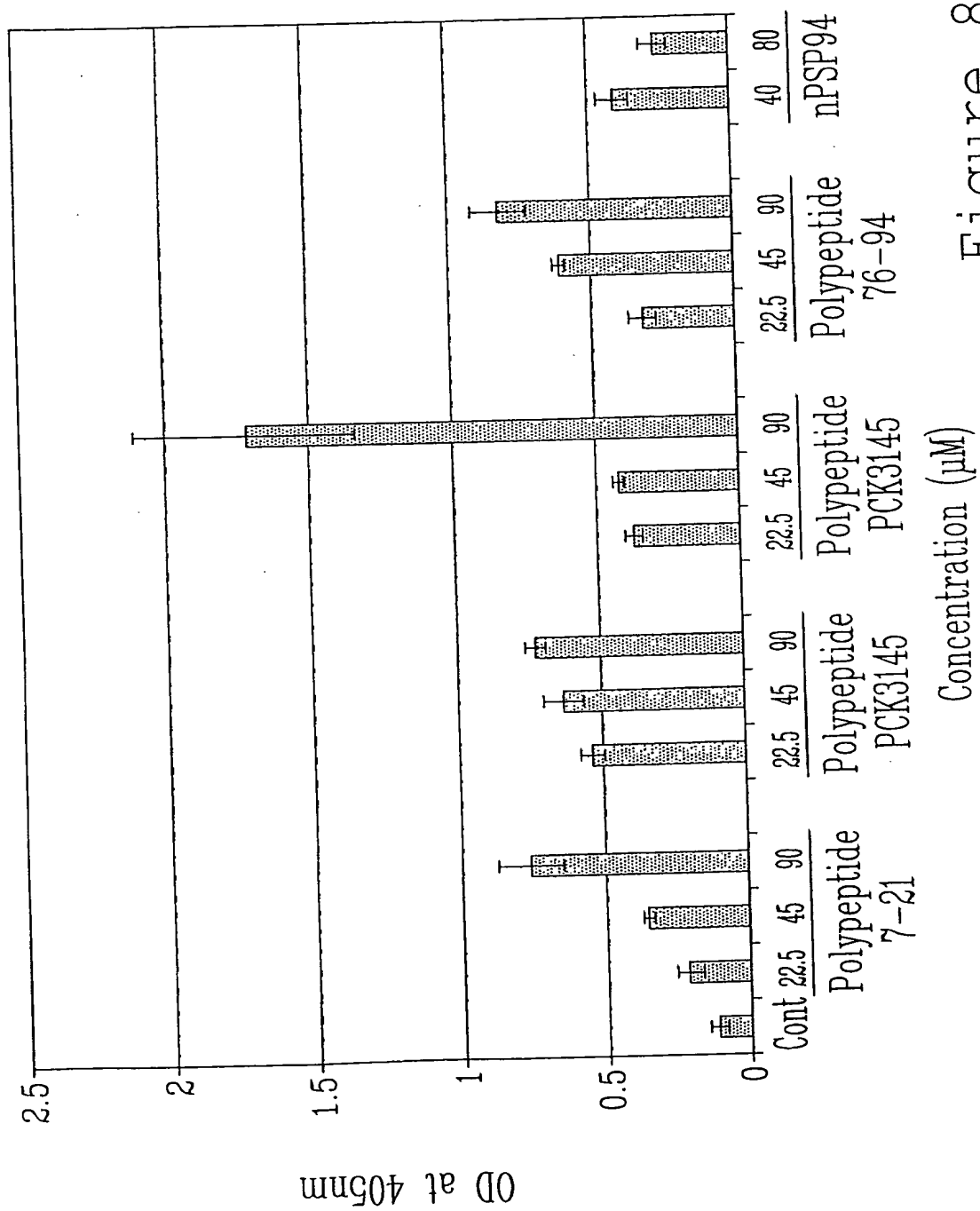


Figure 8

9/30

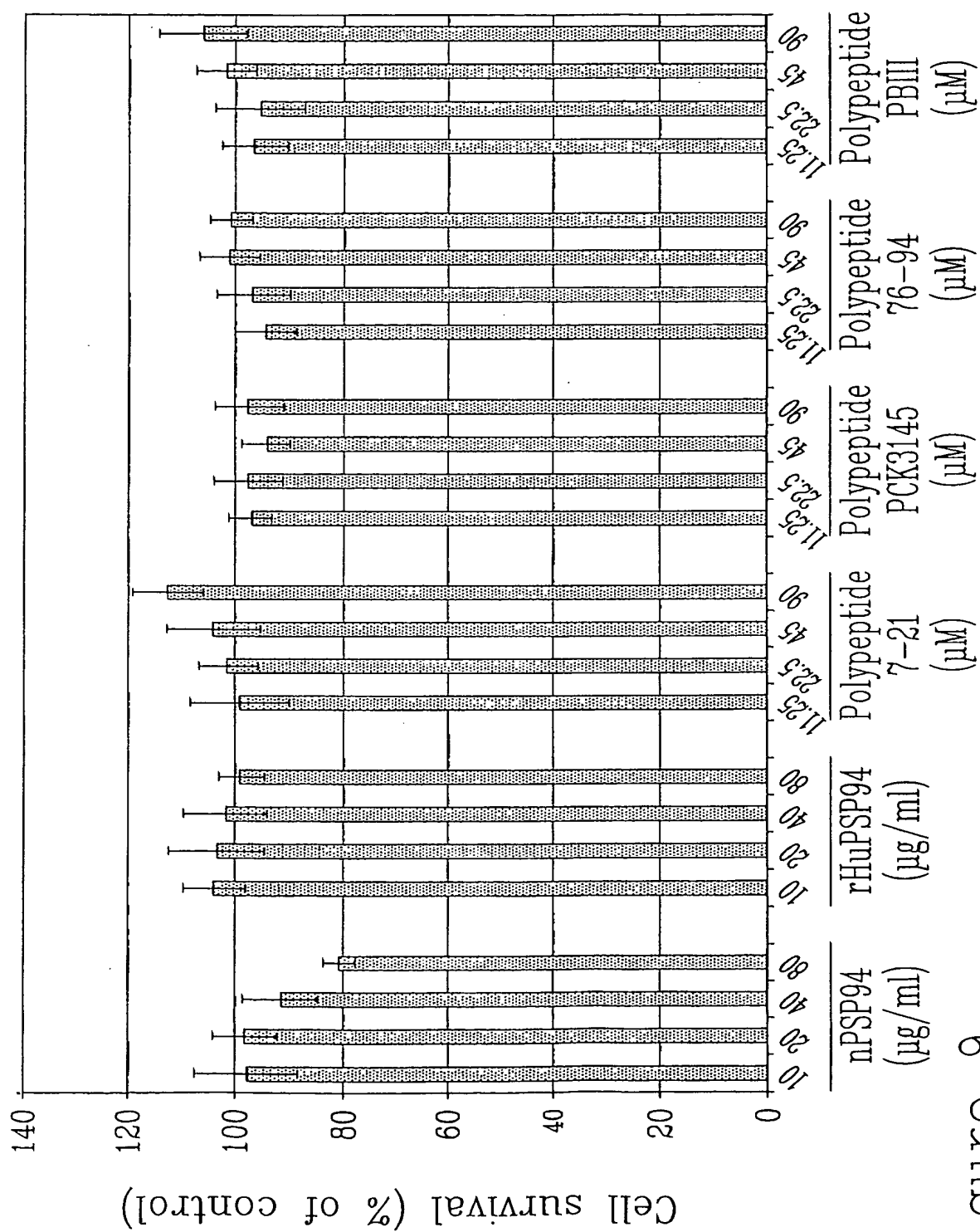


Figure 9

10/30

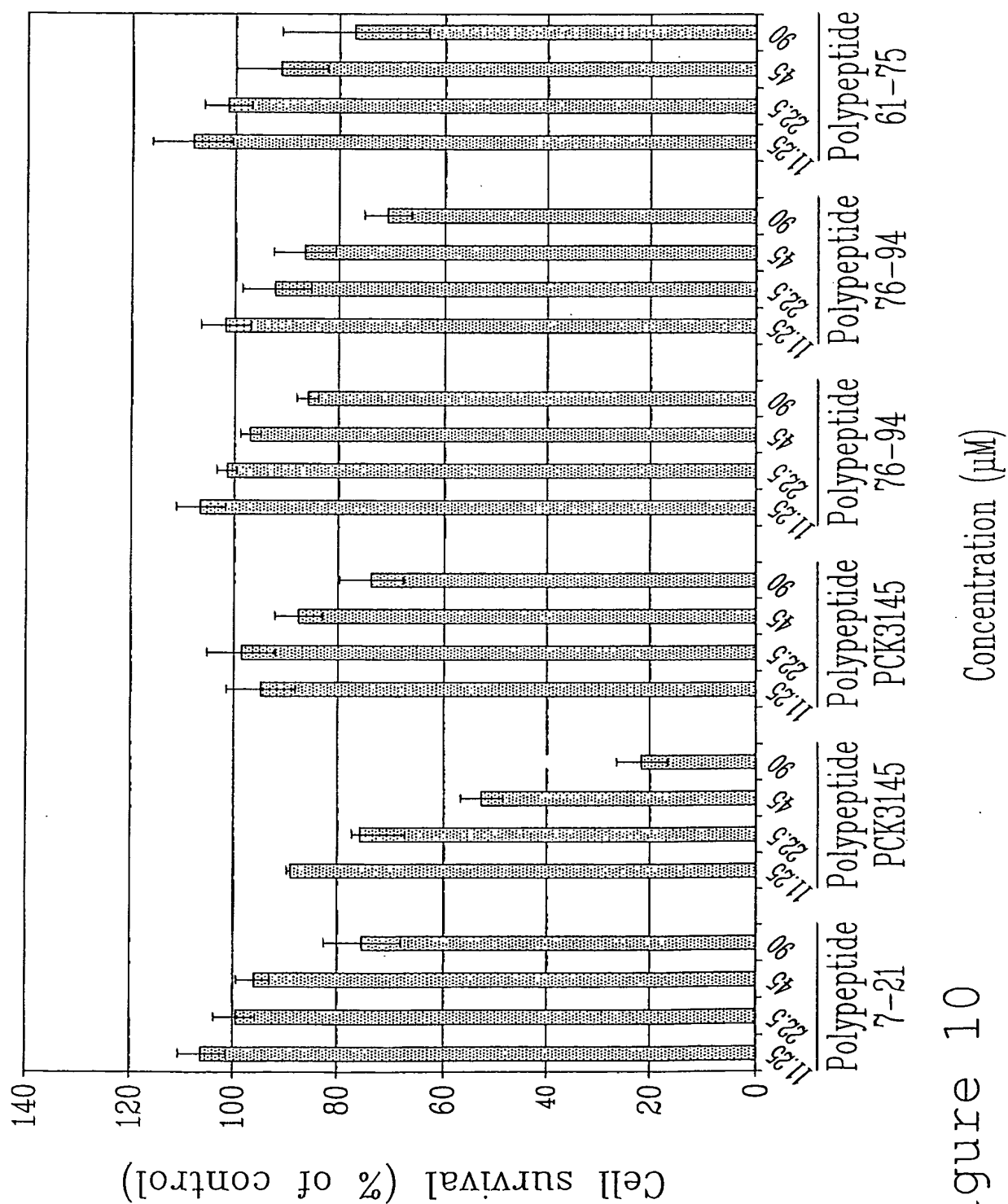


Figure 10

11/30

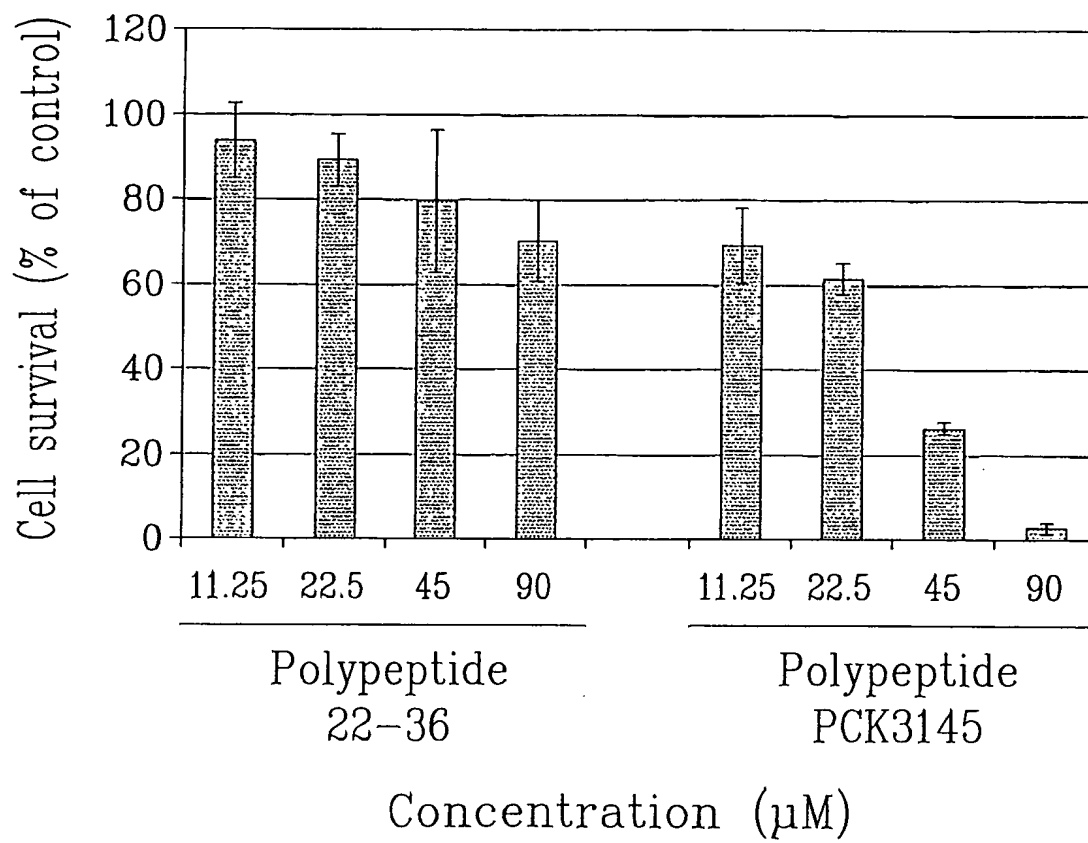
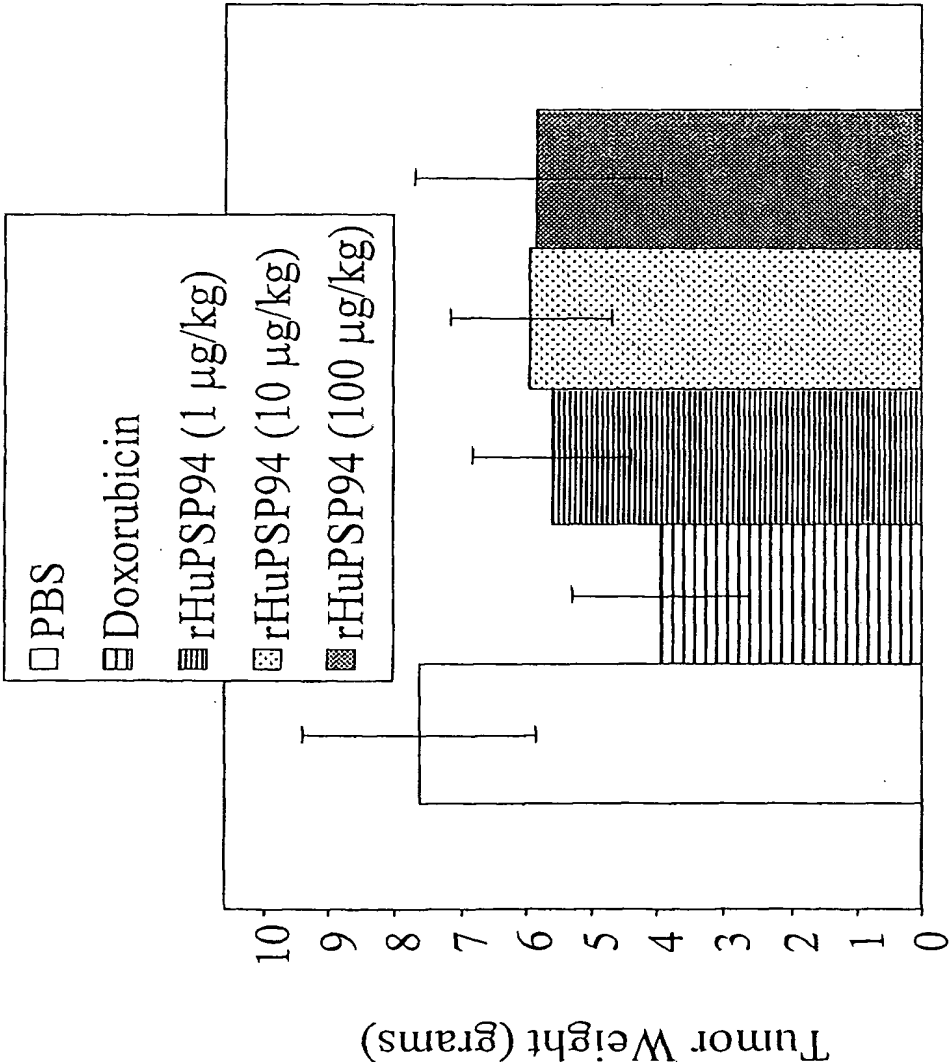


Figure 11

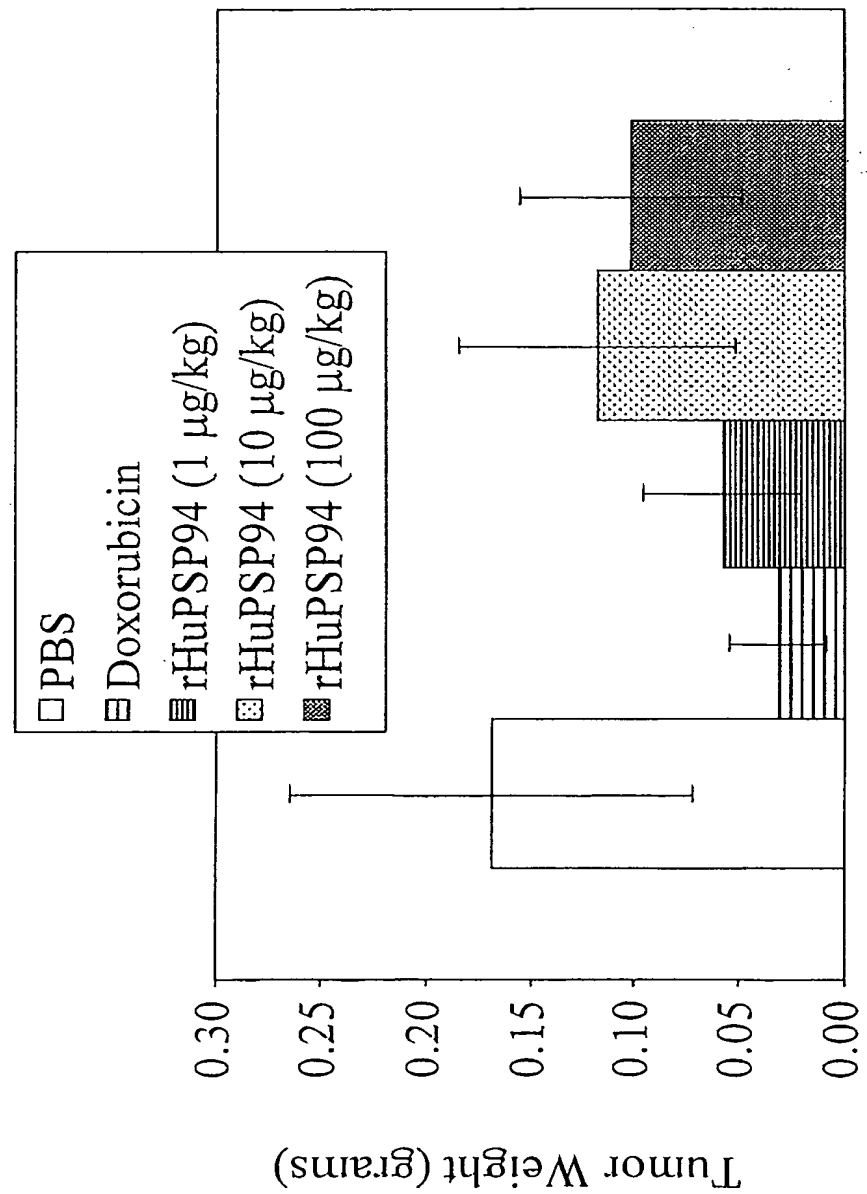
Figure 12



Average Tumor Weights at Day 14 p.t.i.

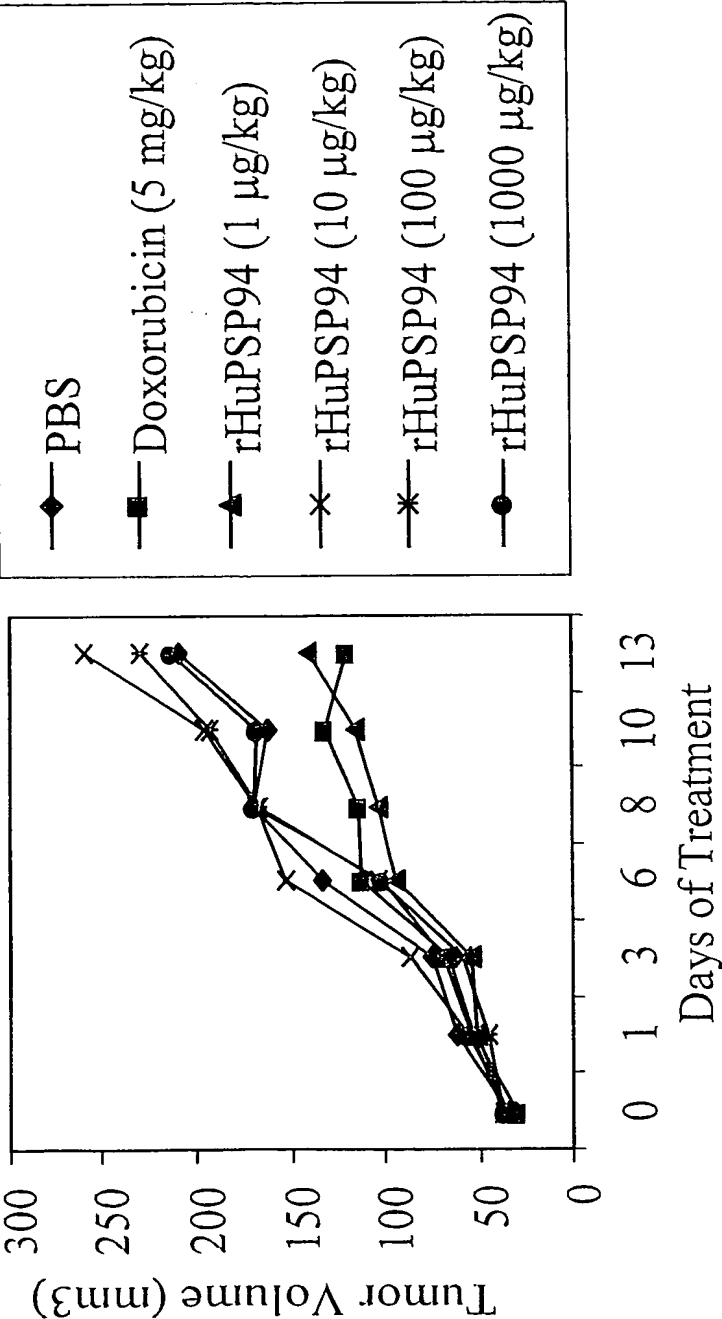
13/30

Figure 13



Average Tumor Weights at Day 14 p.t.i.

Figure 14



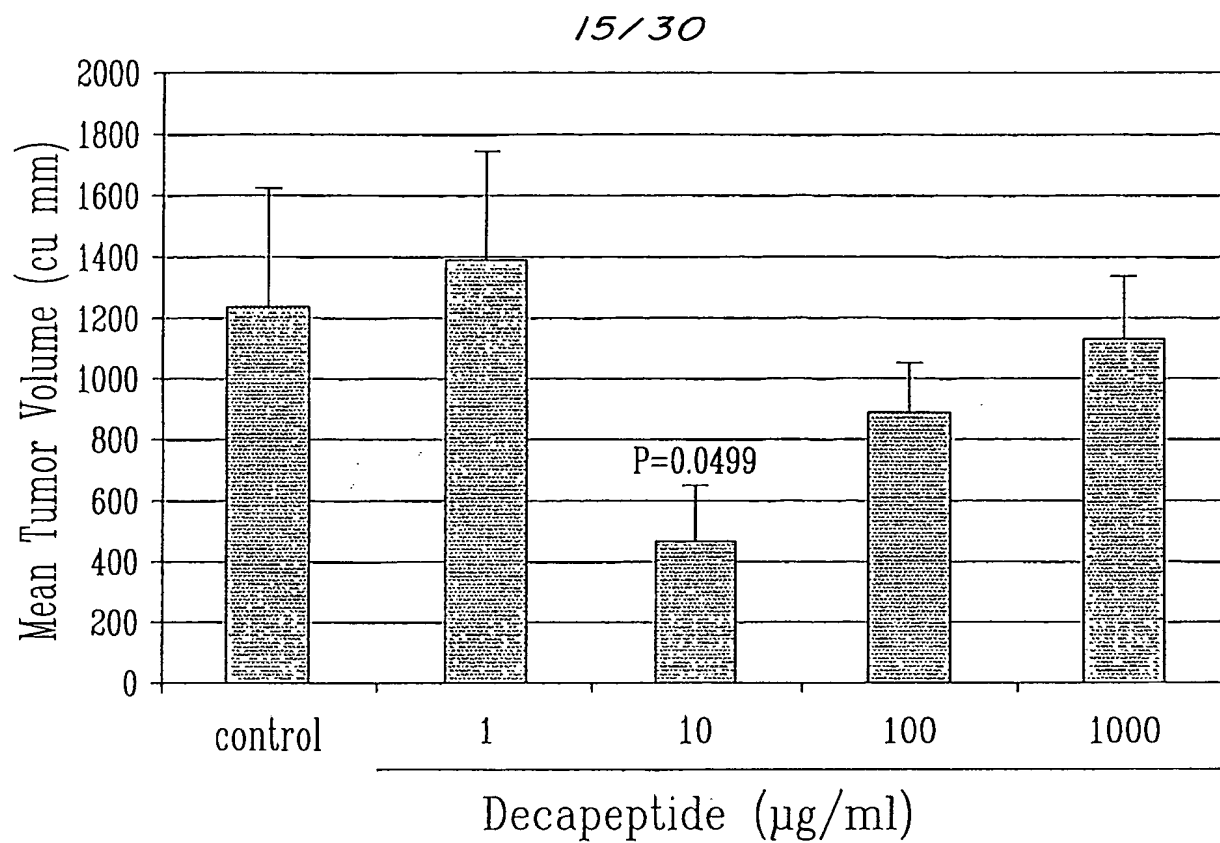


Figure 15

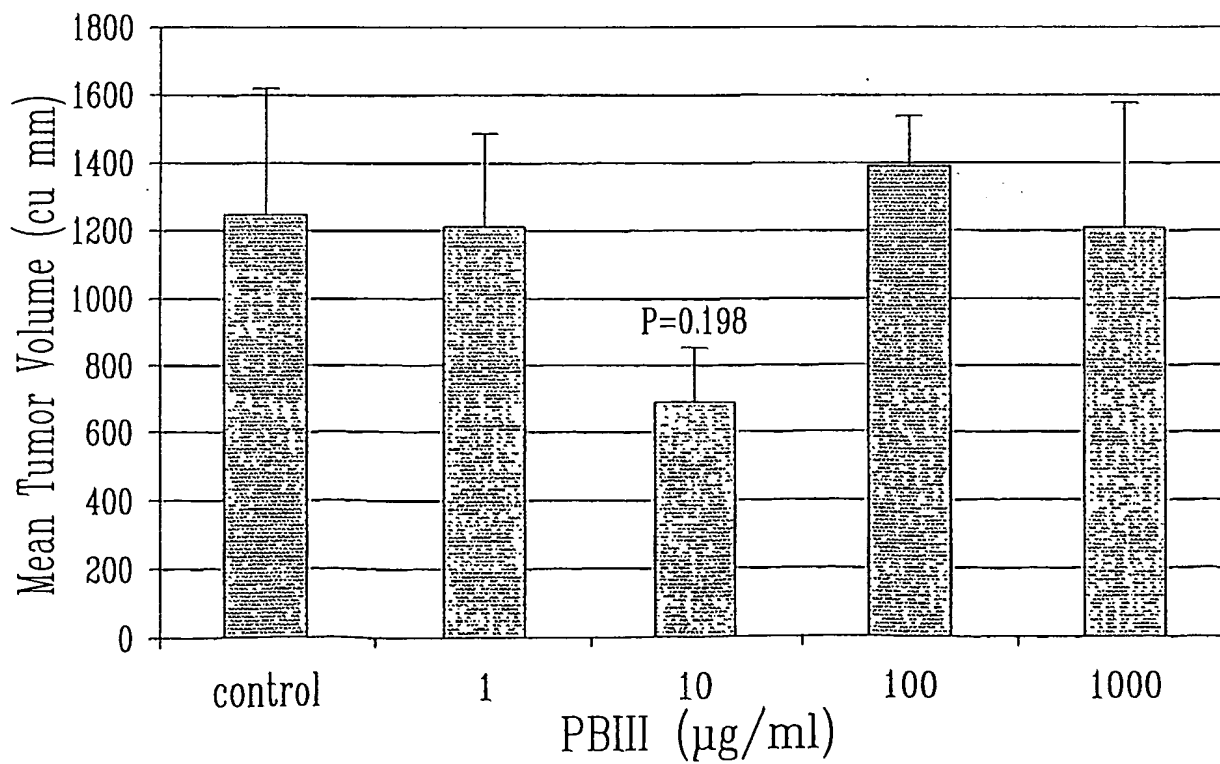


Figure 16

16/30

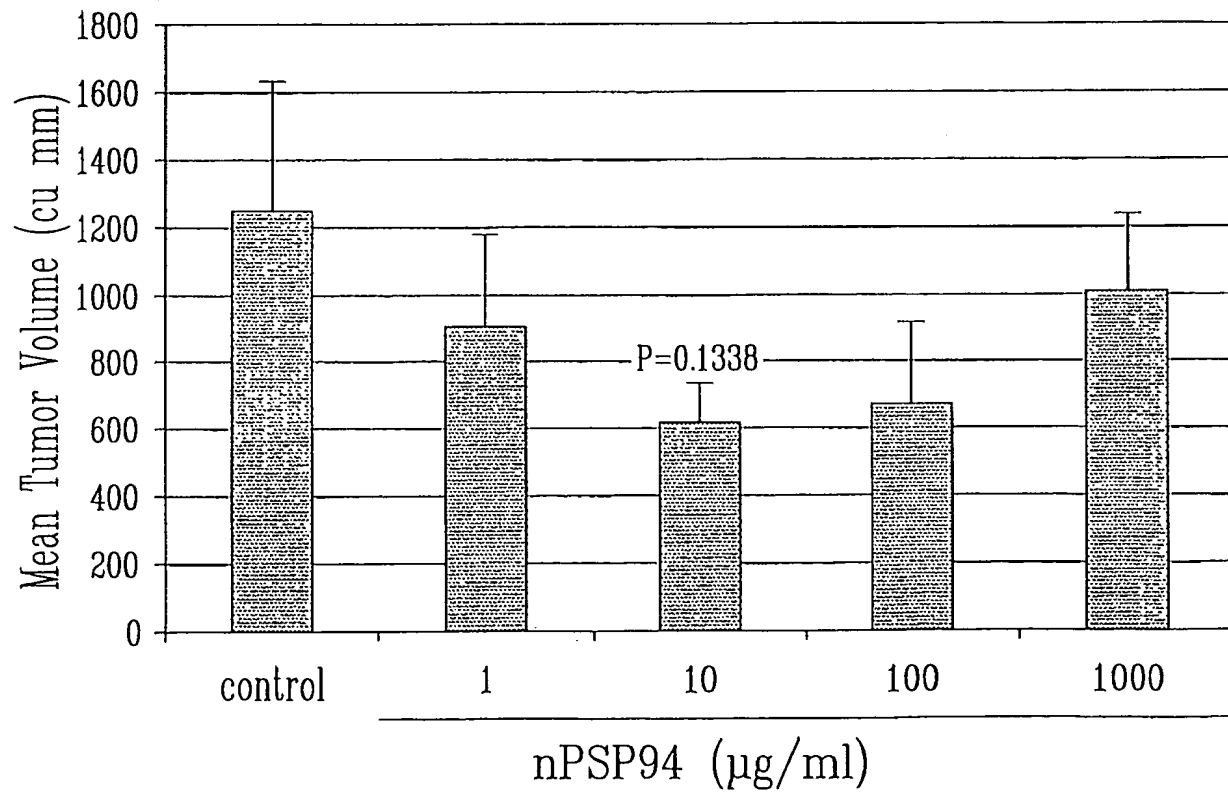
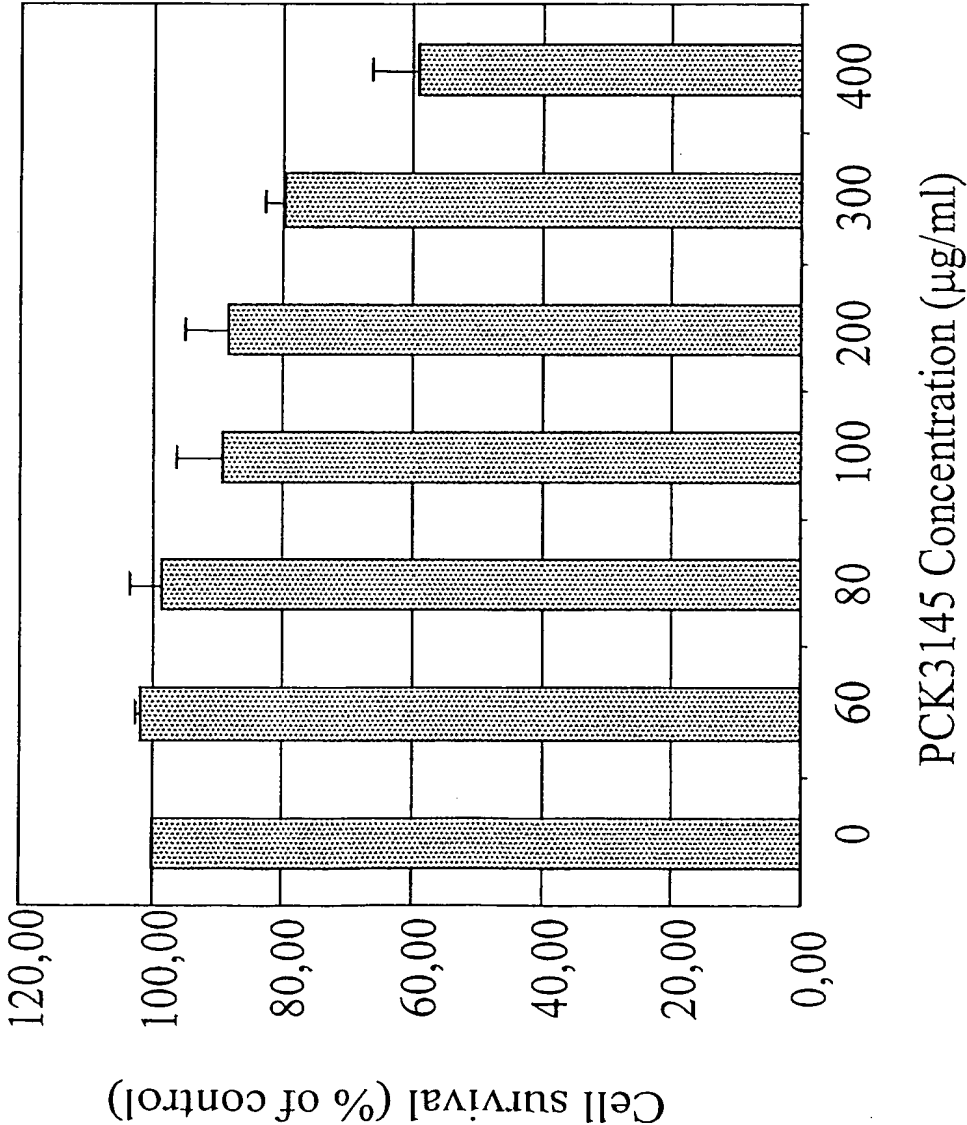


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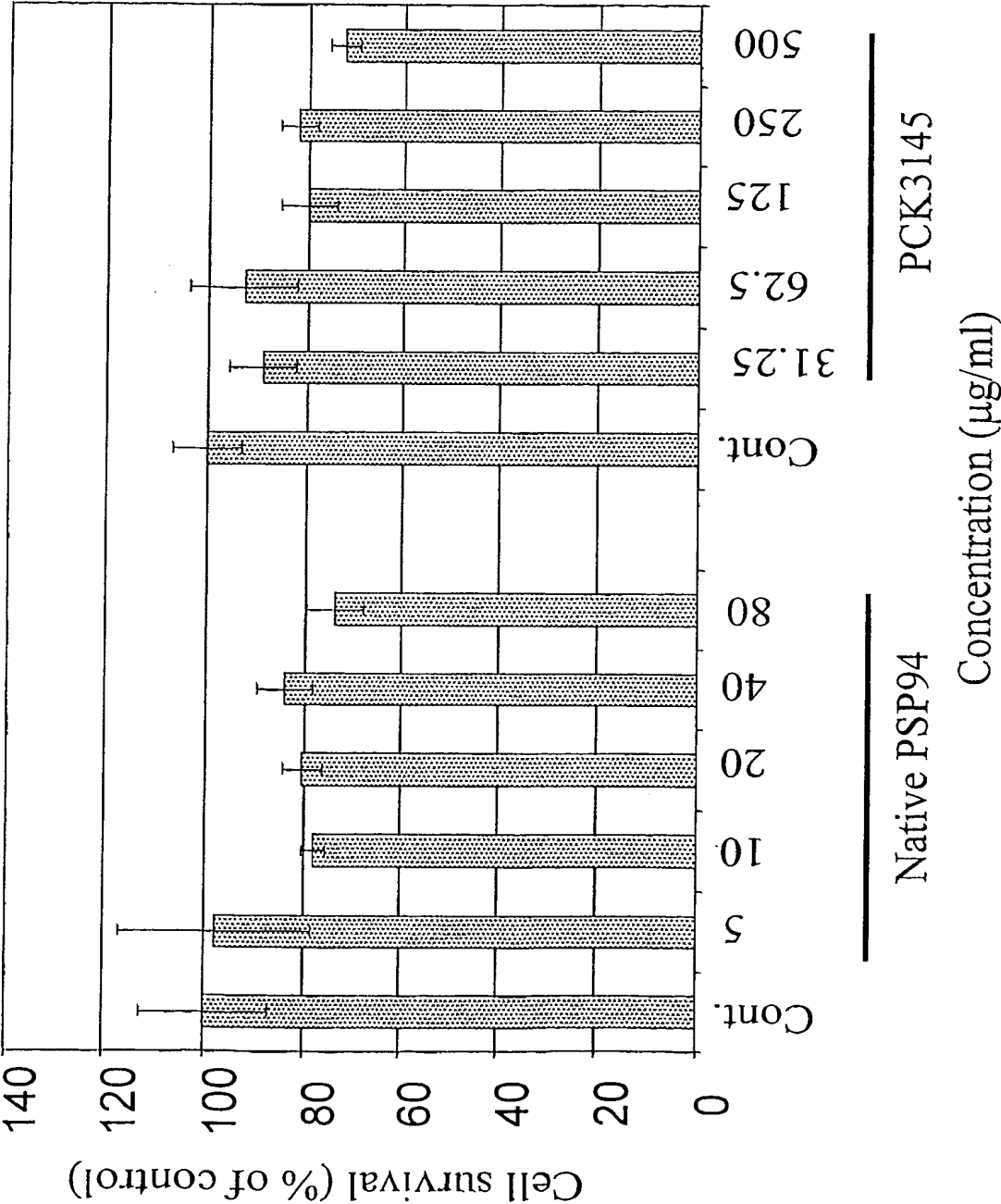
17/30

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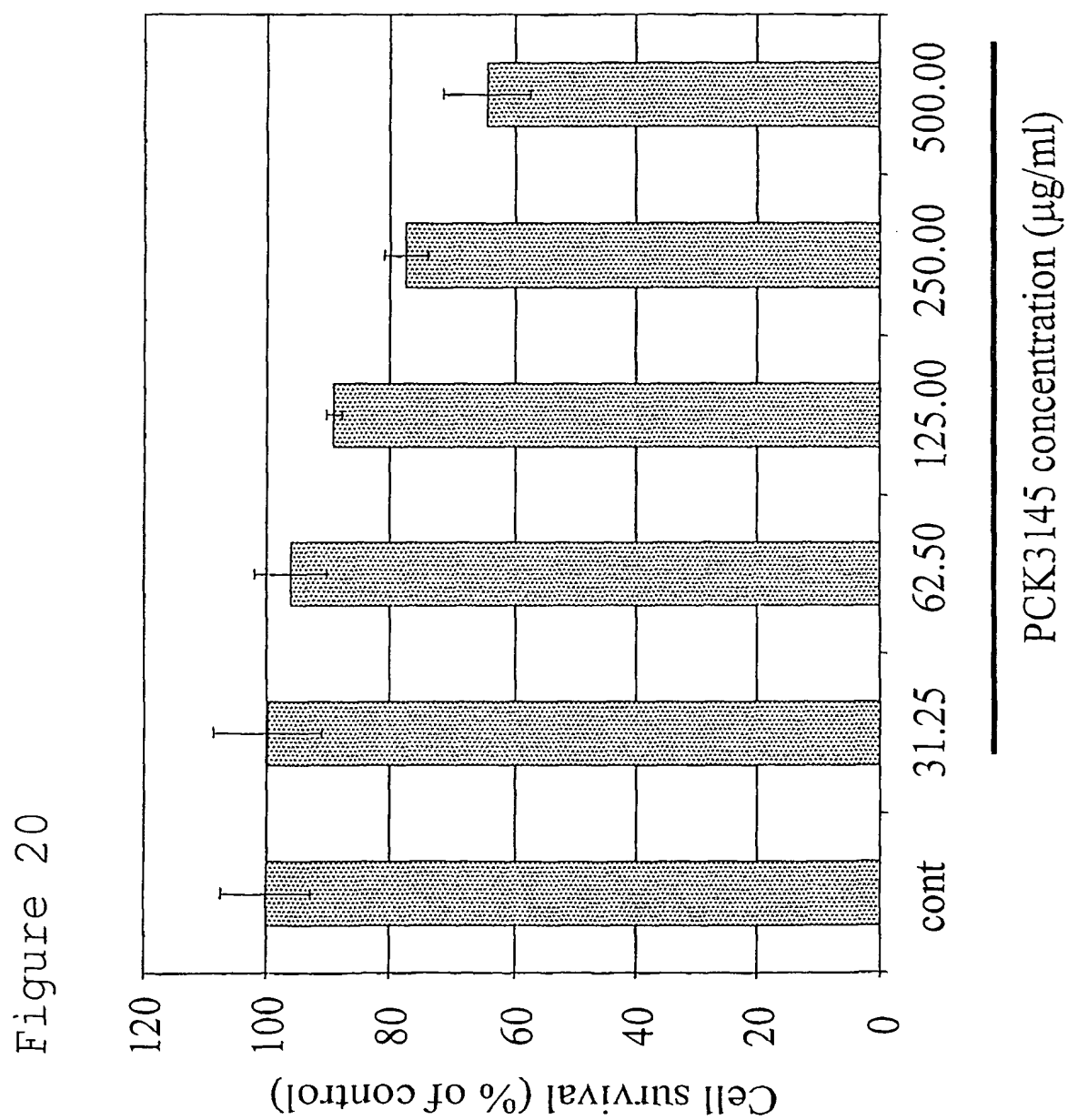


18/30

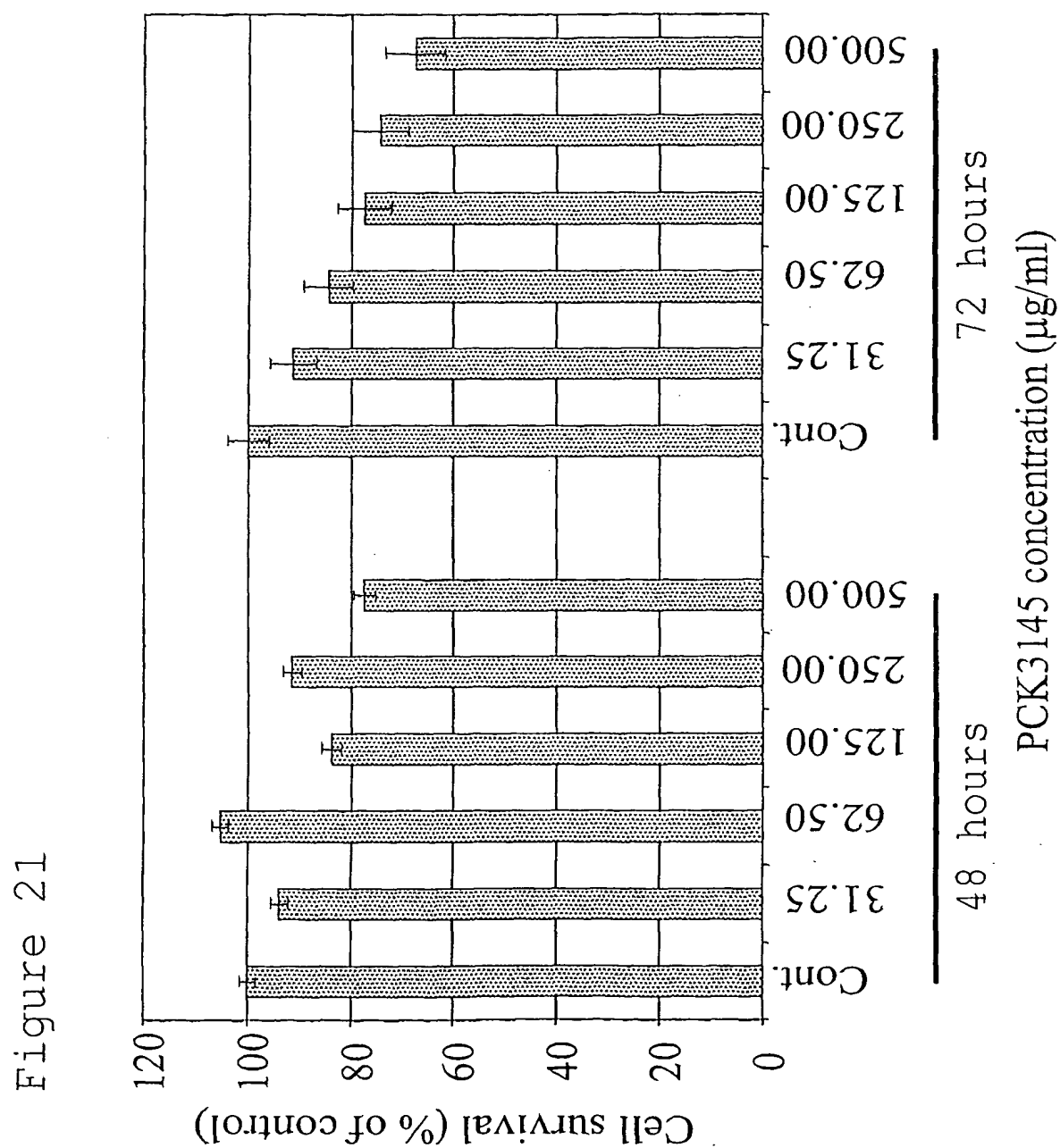
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19/30

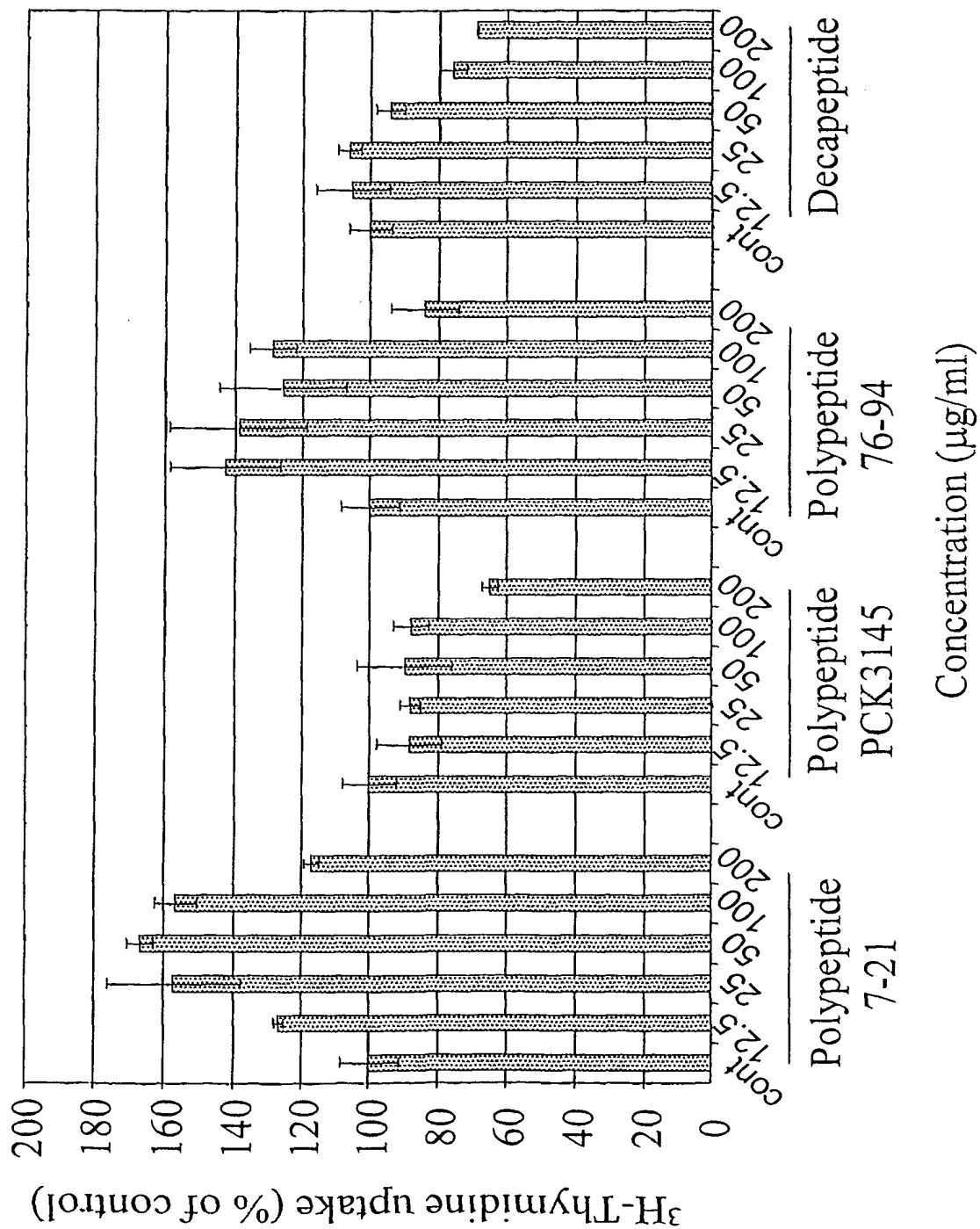


20/30



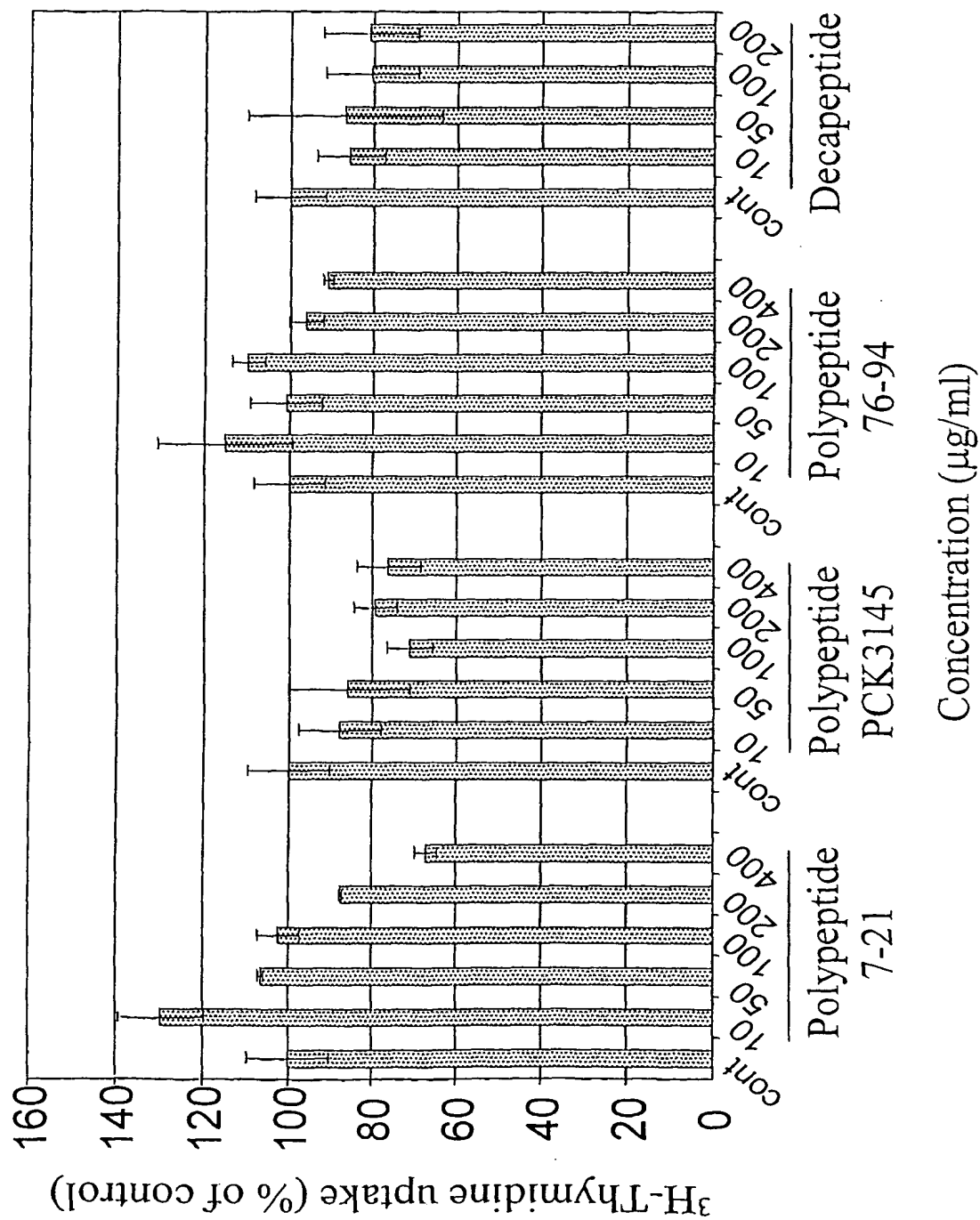
21/30

Figure 22



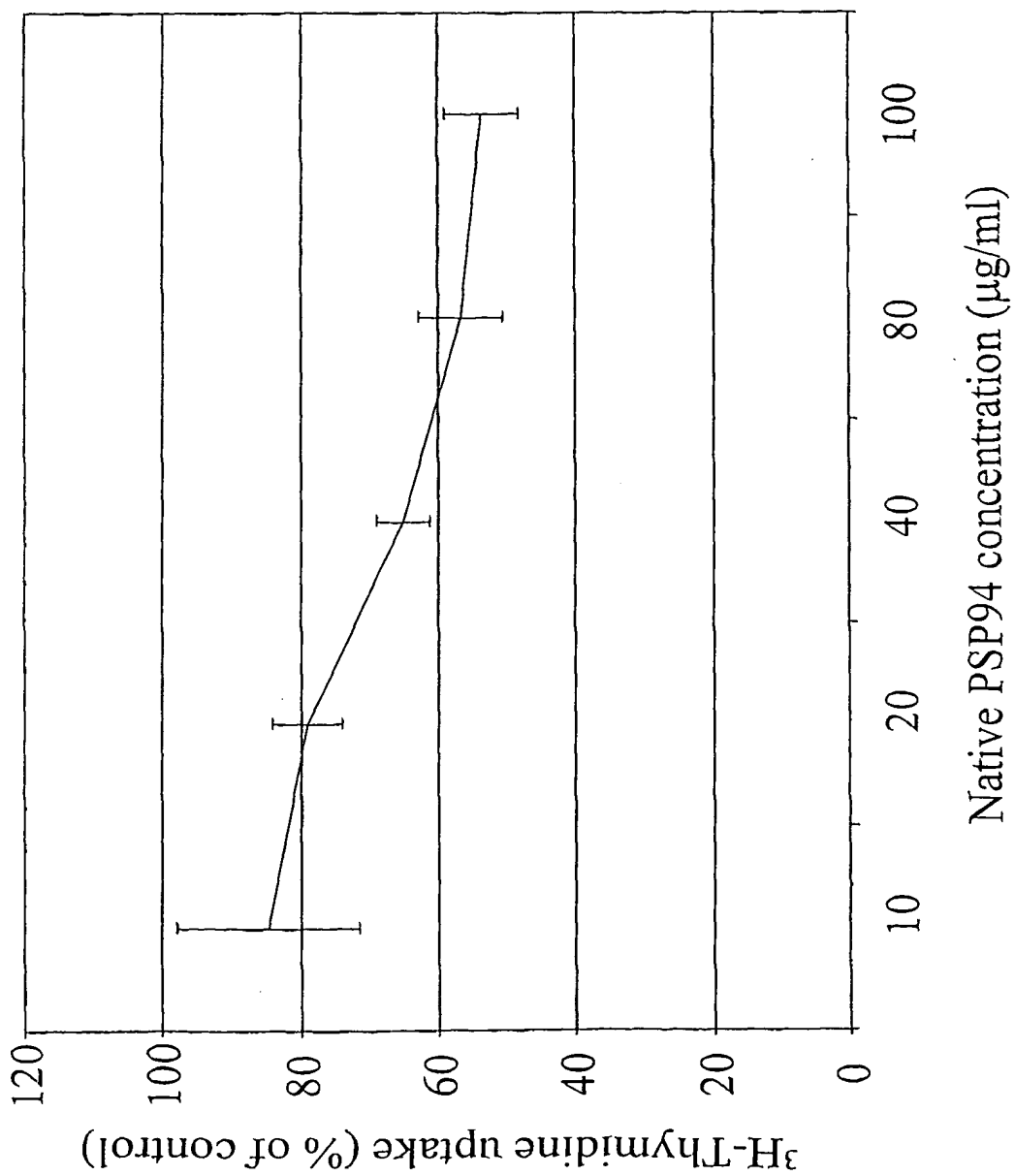
22/30

Figure 23



23/30

Figure 24



24 / 30

1 2 3 4

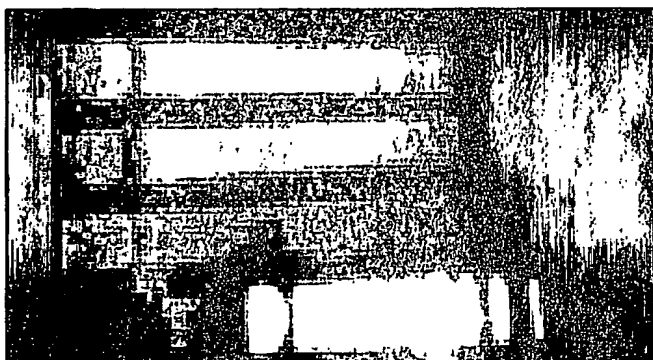


Figure 25

Figure 26

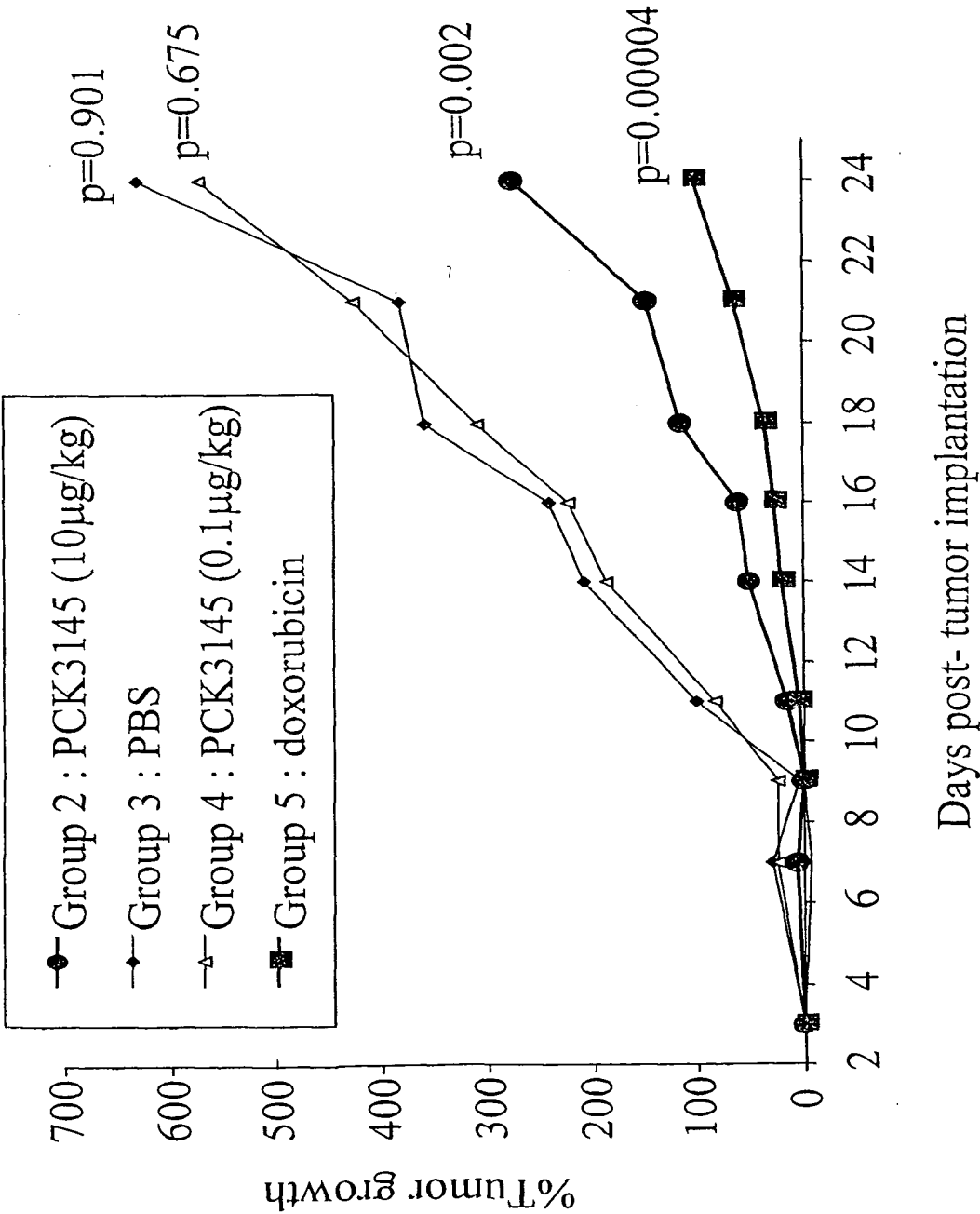
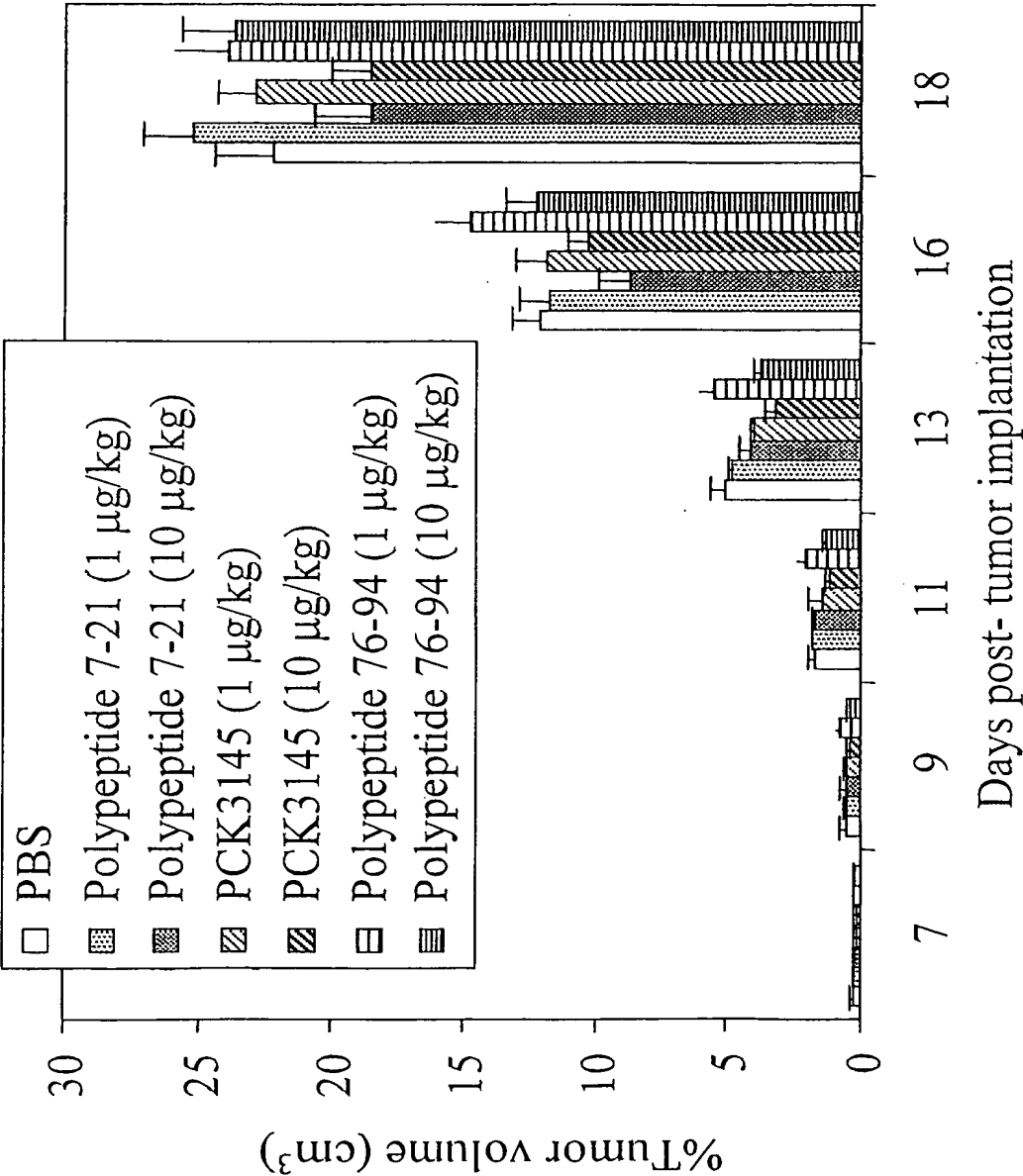
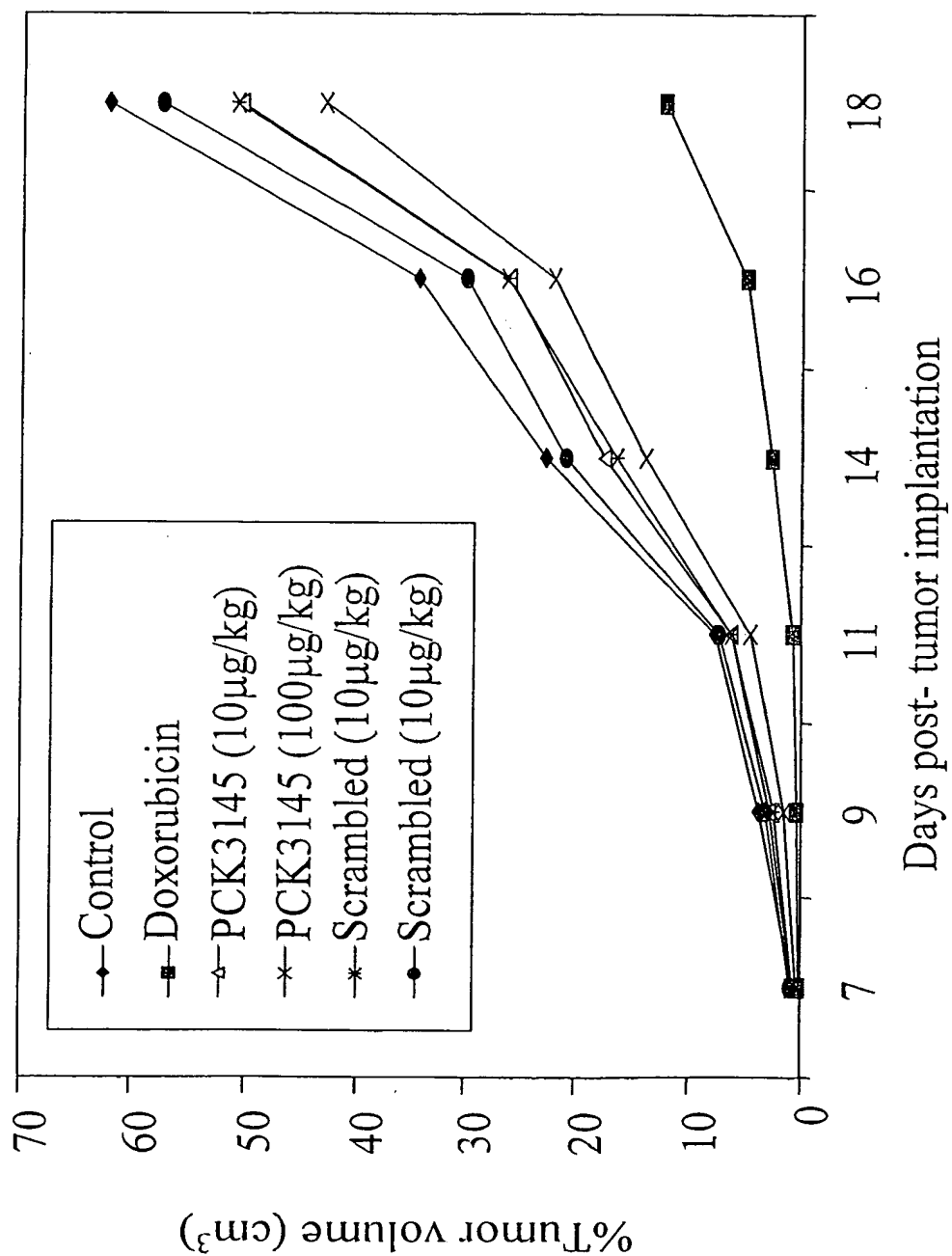


Figure 28



28/30

Figure 29



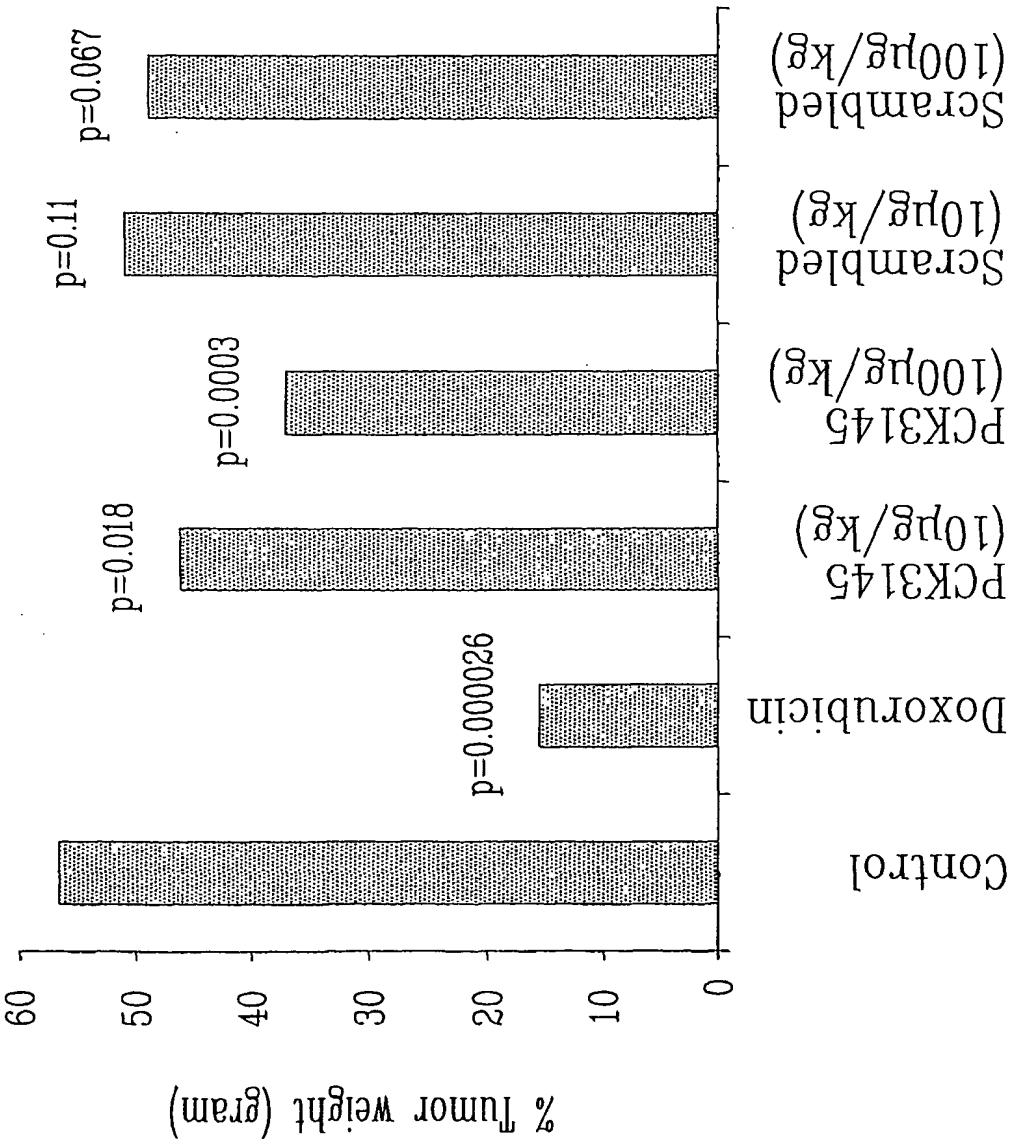
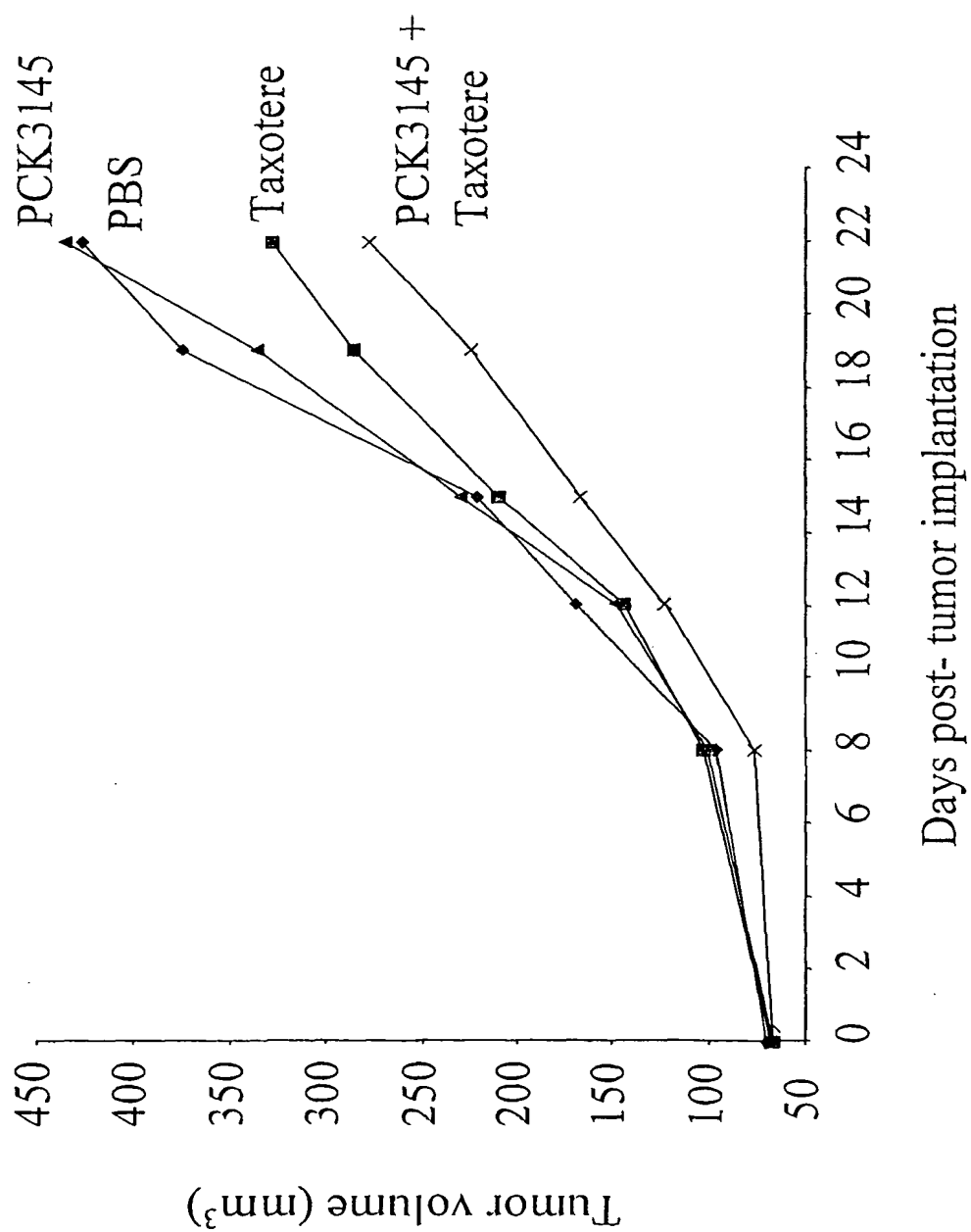


Figure 30

30/30

Figure 31



SEQUENCE LISTING

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 <151> 2000-10-16
 <150> 2,355,334
 <151> 2001-08-20
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Gln	Arg	Ile	Phe	Lys	Lys	Glu	Asp	Cys	Lys	Tyr	Ile	Val	Val	Glu	Lys
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Lys	His	Pro	Ile	Asn	Ser	Glu	Trp	Gln	Thr	Asp	Asn	Cys	Glu	Thr	Cys
			35				40					45			
Thr	Cys	Tyr	Glu	Thr	Glu	Ile	Ser	Cys	Cys	Thr	Leu	Val	Ser	Thr	Pro
	50					55					60				
Val	Gly	Tyr	Asp	Lys	Asp	Asn	Cys	Gln	Arg	Ile	Phe	Lys	Lys	Glu	Asp
65					70					75					80
Cys	Lys	Tyr	Ile	Val	Val	Glu	Lys	Lys	Asp	Pro	Lys	Lys	Thr	Cys	Ser
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 Asn Cys Gln Arg Ile Phe
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 Asn Cys Gln Arg Ile Phe Lys
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Asn	Cys	Gln	Arg	Ile	Phe	Lys	Lys	Glu	Asp	Cys					
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Asn Cys Gln Arg Ile Phe Lys Lys Glu Asp Cys Lys Tyr
 35 40 45

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Asn Cys Gln Arg Ile Phe Lys Lys Glu Asp Cys Lys Tyr Ile
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Asn Cys Gln Arg Ile Phe Lys Lys Glu Asp Cys Lys Tyr Ile Val
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 Asn Cys Gln Arg Ile Phe Lys Lys Glu Asp Cys Lys Tyr Ile Val Val
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Glu Lys Lys Asp Pro Lys
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			20					25					30		
Asn	Cys	Gln	Arg	Ile	Phe	Lys	Lys	Glu	Asp	Cys	Lys	Tyr	Ile	Val	Val
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			20					25					30		
Asn	Cys	Gln	Arg	Ile	Phe	Lys	Lys	Glu	Asp	Cys	Lys	Tyr	Ile	Val	Val
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			20					25					30		
Asn	Cys	Gln	Arg	Ile	Phe	Lys	Lys	Glu	Asp	Cys	Lys	Tyr	Ile	Val	Val
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Glu Lys Lys Asp Pro Lys Lys Thr Cys Ser Val Ser Glu Trp
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20 25 30

Asn Cys Gln Arg Ile Phe Lys Lys Glu Asp Cys Lys Tyr Ile Val Val
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Glu Lys Lys Asp Pro Lys Lys Thr Cys Ser Val Ser Glu Trp Ile
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20 25 30

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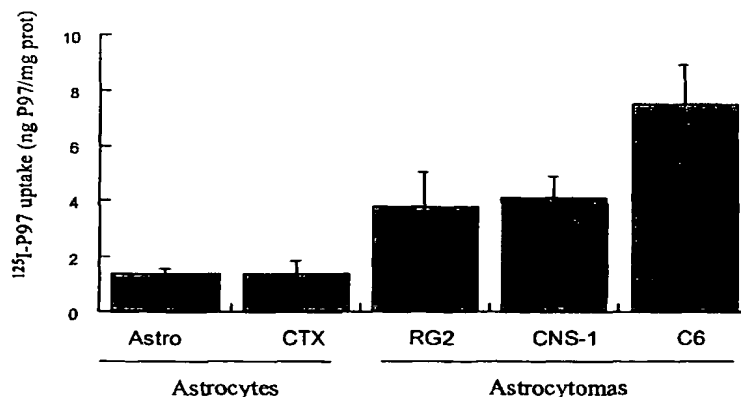
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[Continued on next page]

(54) Title: COMPOSITIONS AND METHODS FOR MODULATING BLOOD-BRAIN BARRIER TRANSPORT

Specific uptake of P97 in astrocytes and astrocytomas



(57) Abstract: This invention provides conjugates of therapeutic or active agents with melanotransferrin or with other ligands of a melanotransferrin receptor, melanotransferrin receptor modulators, and related compositions and methods for modulating blood-brain barrier transport by providing methods of screening and selecting such conjugates, ligands, and modulators in vitro and in vivo, and methods of use of such conjugates, modulators and ligands in diagnosis and the treatment of diseases, including particularly diseases of the central nervous system or lysosomal storage diseases.



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COMPOSITIONS AND METHODS FOR MODULATING BLOOD-BRAIN BARRIER TRANSPORT

CROSS-REFERENCE TO RELATED APPLICATIONS

[01] This application claims priority of U.S. Patent Application No. 60/308,002 filed July 25, 2001. The contents of which are each incorporated herein by reference in their entirety.

5

FIELD OF THE INVENTION

[02] The present invention relates to compositions and methods for modulating blood-brain barrier transport of compounds. In addition, the present invention provides screening assays for identifying compounds that are useful for modulating transport across the blood-brain barrier.

10

BACKGROUND OF THE INVENTION

[03] In the early 1980's, melanotransferrin (MTf) was identified as an oncofetal antigen that was either not expressed, or only slightly expressed in normal tissues, but was found in much larger amounts in neoplastic cells (especially malignant melanoma cells) and fetal tissues (Woodbury, et al., P.N.A.S. USA, 77:2183-2187 (1980)). More recently, there have been additional reports of human MTf being identified in normal tissues, including sweat gland ducts, liver endothelial cells and the endothelium and reactive microglia of the brain (Jefferies, et al., Brain Res., 712:122-126 (1996); and Rothenberger, et al., Brain Res., 712:117-121 (1996)). Interestingly, normal serum contains very low levels of soluble circulating MTf, but increased soluble serum MTf has been found in patients with advanced Alzheimer's Disease (Kennard, et al., Nat. Med., 2:1230-1235 (1996); U.S. Patent No. 5,981,194)

15

[04] The biochemical role and metabolism of MTf has proven difficult to elucidate. Based on appearances, MTf is deceptively similar to transferrin (Tf) and lactotransferrin (lactoferrin or Lf). In humans, these proteins share a 37-39% amino acid sequence homology. In particular, each of these proteins reversibly binds iron, and their N-terminal iron binding domains are quite similar (Baker, *et al.*, *TIBS*, 12:350-353 (1987)).

20

[05] However, functional parallels between these proteins have not been confirmed. For one thing, unlike Tf and Lf, MTf exists in both a membrane bound form and a serum soluble form. Further, in contrast to Tf and Lf, no cellular receptor for MTf has been identified. Serum soluble Tf is known to be taken into cells in an energy-dependent process mediated by the transferrin receptor (Tf-R) (Cook, *et al.*, *Annu. Rev. Med.*, 44:63-74 (1993)). Lf internalization is also likely to be mediated by a receptor mediated process (Fillebeen, *et al.*, *J. Biol. Chem.*, 274(11):7011-7017 (1999)). Two known receptors for Lf are LRP1 and RAGE, although others may exist (Meilinger, *et al.*, *FEBS Letters*, 360:70-74 (1995); Schmidt, *J. Biol. Chem.*, 269(13):9882-9888 (1994)).

[06] Although it has been postulated that MTf is an alternate ligand for Tf-R (*see*, U.S. Patent No. 5,981,194), no published data confirms this finding. Further, although various studies have confirmed iron transport into cells by membrane bound MTf, it occurs only in cells where membrane bound MTf is overexpressed well beyond physiological levels (Richardson, *Eur. J. Biochem.*, 267:1290-1298 (2000)).

[07] Therapeutic and diagnostic agents conjugated to soluble MTf are the basis of recently filed U.S. Provisional Patent Application Nos. 60/226,242 and 60/226,254, the teachings of which are incorporated herein by reference for all purposes.

[08] The blood-brain barrier (BBB) performs a neuroprotective function by tightly controlling access to the brain; consequently it also impedes access of pharmacological agents to cerebral tissues, necessitating the use of vectors for their transit. Blood-brain barrier (BBB) permeability is frequently a rate-limiting factor for the penetration of drugs or peptides into the central nervous system (CNS) (see Pardridge, W.M. *J. Neurovirol.* 5: 556-569 (1999); Bickel, U., Yoshikawa, T. & Pardridge, W.M. *Adv. Drug Deliv. Rev.* 46: 247-279 (2001)). The brain is shielded against potentially toxic substances by the BBB, which is formed by brain capillary endothelial cells that are closely sealed by tight junctions. In addition, brain capillaries possess few fenestrae and few endocytic vesicles, compared to the capillaries of other organs (see Pardridge, W.M. *J. Neurovirol.* 5: 556-569 (1999)). There is little transit across the BBB of large, hydrophilic molecules aside from some specific proteins such as transferrin, lactoferrin and low-density lipoproteins, which are taken up by receptor-mediated endocytosis (see Pardridge, W.M. *J. Neurovirol.* 5: 556-569 (1999); Tsuji, A. & Tamai, I. *Adv. Drug Deliv. Rev.* 36: 277-290 (1999); Kusuhara, H. & Sugiyama, Y. *Drug Discov. Today* 6:150-156 (2001); Dehouck, B. *et al.* *J. Cell. Biol.* 138: 877-889 (1997); and Fillebeen, C. *et al.* *J. Biol. Chem.* 274: 7011-7017 (1999)).

[09] In order to understand and improve the delivery of therapeutic agents into cells, it is highly desirable to understand the receptors and metabolic basis of MTf activity. It is an object of this invention to identify the receptor for MTf, and to provide methods and compounds for improving the delivery of therapeutic and diagnostic agents into cells, in particular, therapeutic and diagnostic agents conjugated to MTf, and particularly their delivery across the blood-brain barrier.

BRIEF SUMMARY OF THE INVENTION

[10] The present invention provides compositions and methods for modulating transcytosis, endocytosis, and blood-brain barrier transport of compounds. In addition, the present invention provides screening assays for identifying compounds that are useful for modulating transport across the blood-brain barrier and for delivering active agents conjugated to such agents which undergo endocytosis or transcytosis upon binding to the LRP (e.g., LRP1 and LRP1B) receptor.

[11] In one aspect, the invention provides methods for screening compounds for the ability to modulate the endocytosis or transcytosis of p97 and p97 conjugated to active agents by assessing their ability to bind a low density lipoprotein receptor related protein (LRP). In another aspect the invention, provides modulators of the endocytosis or transcytosis of p97 and p97 conjugated to active agents. In another aspect, the invention provides conjugates of compounds (e.g., ligands) which bind to the LRP receptor and which undergo endocytosis or transcytosis upon binding to the LRP receptor. In another aspect, the invention provides methods of determining the capability of a given cell, cell type, or tissue to endocytose or transcytose p97 or a p97 conjugate to an active agent by measuring the expression of an LRP receptor nucleic acid or protein of the cell, cell type, or tissue.

[12] In another aspect, the invention provides ligands and modulators of the LRP family of receptors for use as modulators of p97 transcytosis. In another aspect, the invention provides conjugates of LRP receptor ligands for use in delivering therapeutic agents across the blood brain barrier and/or to an intracellular compartment, particularly the lysosome. In another aspect, the invention provides methods for modulating the expression of LRP receptors by administering p97.

[13] In one embodiment, the receptor is the LRP1 receptor. In a preferred embodiment, the receptor is the LRP1B receptor.

[14] In one embodiment, the present invention provides a method for identifying a compound that modulates or binds to a melanotransferrin receptor ("MTf-R" or "p97 receptor"), the method comprises contacting the compound with the p97 receptor and determining the effect of LRP receptor ligands (e.g., lactoferrin, p97, BSA, aprotinin, and

5 RAP) on the interaction of the compound with the receptor. In another embodiment, one or more LRP receptor ligands (e.g., lactoferrin, RAP, BSA, aprotinin, and p97) are individually contacted with the p97 receptor and the effect of the compound on the interaction of each compound with the p97 receptor is respectively determined. In one embodiment, the functional effect is an effect on transcytosis. In another embodiment, the effect is on
10 endocytosis. In another embodiment, the effect is on receptor binding as measured by the changes in the binding, for instance, of RAP, p97, or lactoferrin to the p97 receptor. In certain embodiments, the method is a high throughput screening assay. In a preferred embodiment, the receptor is the LRP1 receptor. In a more preferred embodiment, the receptor is the LRP1B receptor.

15 [15] In one embodiment, the invention provides a method for identifying a selective modulator of p97 receptor activity, by contacting the modulator and a ligand of the LDL-R receptor family ligand with the melanotransferrin receptor and determining the ability of one to affect the binding or interaction of the other with the melanotransferrin receptor. In a preferred embodiment, the LDL-R receptor ligand is selected from PAI-1 (plasminogen
20 activator inhibitor type-1), plasminogen, pro-uPA (pro-urokinase plasminogen activator), tissue factor inhibitor, tPA (tissue type plasminogen activator), activated α_2 -macroglobulin, α_1 -chymotrypsin, cathepsin G, lactoferrin, RAP (receptor associated protein), thyroglobulin, circumsporozite protein, saposin, gentamycin, polymixin B, pseudomonas exotoxin A, seminal vesicle secretory protein A, thrombospondin -1, β -VLDL, chylomicron remnants,
25 IDL, Lp(a), VLDL (very low density lipoprotein), ApoB100 (apolipoprotein B 100), and Apolipoprotein E (Apo E). In a further embodiment, a plurality of such ligands are selected and the selectivity of the compound for the receptor is assessed according to how similarly to p97 the compound's interaction with the receptor is affected by such ligands. In a further embodiment, the LDL-R receptor ligand is a LRP receptor ligand. In a more preferred
30 embodiment, the LRP receptor ligand is an LRP1 receptor ligand or an LRP1B receptor ligand. In one embodiment, the effect on binding is assessed indirectly by a functional effect (e.g., transcytosis, endocytosis). In another embodiment, the effect is on receptor binding as measured by the changes in the binding, for instance, of RAP, p97, or lactoferrin from the

p97 receptor. In certain embodiments, the method is a high throughput screening assay. In a further embodiment, the method is a BioCore method. In a preferred embodiment, the receptor is the LRP1 receptor. In a more preferred embodiment, the receptor is the LRP1B receptor. In another embodiment, the screening assay is measures the competitive
5 displacement of a ligand, preferably p97, of the p97 receptor,

[16] In one embodiment, the melanotransferrin receptor modulator or ligand has neurological activity such that it is useful in the treatment, prophylaxis or diagnosis of a neurological disorder. In other embodiments, the melanotransferrin receptor modulator or ligand is useful for the modulation of the uptake of melanotransferrin conjugated therapeutic
10 agents into the brain. In still other embodiments, the compound is useful for reducing a neurological side-effect of such a therapeutic agent. In a preferred embodiment, the disease or disorder is Alzheimer's disease.

[17] In one embodiment, the p97 modulatory compound has neurological activity such that it is useful in the treatment, prophylaxis or diagnosis of a neurological disorder. In other
15 embodiments, the compound is useful for the modulation of the uptake of melanotransferrin conjugated therapeutic agents into the brain. In still other embodiments, the compound is useful for reducing a neurological side-effect of a therapeutic agent. In another embodiment, the present invention provides a method of treating a neurological disorder in a patient, the method comprising administering to the patient a therapeutically effective amount of the
20 modulatory compound.

[18] In one embodiment, the p97 conjugate or p97 receptor ligand-conjugate comprises a therapeutic agent useful in treating a lysosomal storage disease. In one embodiment, the therapeutic agent is an enzyme deficient in a patient having such a disorder. In one
25 embodiment the enzyme is iduronidase. In another embodiment, the present invention provides a method of treating a lysosomal storage disease in a patient, the method comprising administering to the patient with a lysosomal storage disease a therapeutically effective amount of p97 conjugated to an enzyme with an activity which is deficient in the lysosomal storage disease. In one embodiment, a modulator or LRP or LRP1B is co-administered to modulate the therapeutic or adverse effects of such a conjugate. In one embodiment, the
30 conjugate is a fusion protein comprising a p97 portion and an enzyme portion wherein the enzyme provides the enzymatic activity deficient in the lysosomal storage disease. In one embodiment the enzyme is α -L-iduronidase. In one embodiment, the p97 portion is a

fragment of p97 sufficient for the endocytosis or transcytosis of the conjugate or fusion protein.

[19] In one embodiment, the p97 conjugate or p97 receptor modulator-conjugate comprises a therapeutic agent useful in treating a CNS tumor such as a glioblastoma. In one embodiment, the therapeutic agent is a cancer chemotherapeutic agent. In another embodiment, the present invention provides a method of treating a patient with a brain or CNS tumor or glioblastoma by administering to the patient a therapeutically effective amount of p97 conjugated to the chemotherapeutic agent. In a preferred embodiment, the conjugate binds to the LRP1B receptor. In one embodiment, a modulator of LRP or LRP1B is co-administered to modulate the therapeutic or adverse effects of such a conjugate.

[20] In another embodiment, the present invention provides a method of modulating a melanotransferrin receptor ("MTf-R"), the method comprises contacting the MTf-R with a modulator identified using the above method.

[21] In yet another embodiment, the present invention provides a method for increasing the uptake of a melanotransferrin conjugated therapeutic agent into the brain of a patient, the method comprising administering a modulator of MTf-R biological activity and the melanotransferrin conjugated therapeutic agent. In one embodiment, the modulator of MTf-R biological activity and the melanotransferrin conjugated therapeutic agent are administered contemporaneously. In another embodiment, the modulator of MTf-R biological activity and the melanotransferrin conjugated therapeutic agent are administered sequentially.

[22] In still another embodiment, the present invention provides a method of reducing the uptake of a melanotransferrin conjugated therapeutic agent into the brain of a patient, the method comprising administering a modulator of MTf-R biological activity with a melanotransferrin conjugated therapeutic agent, wherein they are administered either contemporaneously or sequentially. In certain embodiments, the modulator is first identified according to the above method.

[23] The present invention also provides modulators of MTf-R biological activity, wherein the modulator is identified using the above method. Preferably, the modulator is useful for reducing a neurological side-effect of a therapeutic agent.

[24] In yet another embodiment, the present invention provides a method of identifying a compound that modulates melanotransferrin-mediated ("MTf-mediated") iron uptake, the method comprising: contacting a cell expressing MTf on its surface with the compound in

the presence of MTf bound to iron ("holo-MTf") and in the absence of transferrin; and determining the amount of iron uptake into the cell. In certain embodiments, the compound increases the amount of iron uptake into the cell. In other embodiments, the compound decreases the amount of iron uptake into the cell.

5 [25] In another aspect, the invention provides pharmaceutical compositions comprising such modulators, conjugates, and ligands and methods of using such pharmaceutical compositions. In one embodiment, the invention provides a pharmaceutical composition comprising a p97 receptor ligand conjugate for delivering an active agent across the blood
10 brain barrier or into an intracellular compartment. The conjugate can be administered in a pharmaceutically acceptable carrier or diluent.

[26] In other embodiments, a fusion protein comprising a peptide ligand of the LRP1 or LRP1B receptor may be used as the conjugate. The conjugate may therefore be a chimeric fusion protein combining a p97 peptide portion with a peptide active agent portion. The fusion protein active agent may be a substance having therapeutic activity such as a growth
15 factor or lymphokine or peptide drug. The active agent may be an enzyme or other protein. In a preferred embodiment, the fusion protein comprises an active agent which is an enzyme that is deficient or has an activity deficient in a lysosomal storage disease. In particular, enzymes such as α -L-iduronidase or N-acetylgalactosamine 4-sulfatase are contemplated. The invention is also directed to embodiments where such a fusion protein is administered to
20 a subject having a lysosomal storage disease. In other embodiments, the enzyme is an enzyme deficient in a human disease such as PKU (e.g., phenylase). In other embodiments, the enzyme is selected for its beneficial effect (e.g. heparinase I to limit the action of heparin). Such conjugates may alternatively be conjugated by synthetic chemistry reactions or joined by linker groups. One of ordinary skill in the art would understand how to make
25 such fusion proteins. See, for instance, U.S. Patent Application No. US 2001/0025026A1, published September 27, 2001. In some embodiments, the fusion protein may be formed from expression of a nucleic acid encoding the full amino acid sequence of the chimeric protein. In other embodiments, the chimeric fusion protein may be formed by a synthetic reaction combining the two peptides portions via a peptide bond directly linking the two
30 portions.

[27] In other embodiments, the conjugate is alternatively formed by non-covalent bonds between the carrier and an antibody to which the active agent is attached.

[28] The invention also relates to a method of delivering an active agent across the blood brain barrier comprising administering a conjugate of p97 or another ligand of the LRP1B receptor which undergoes endocytosis or transcytosis. The compositions of the invention may also be used for delivering an agent across the blood eye or blood placenta barrier or intracellular to a lysosome.

[29] In some embodiments, the conjugate according to the invention does not comprise p97, RAP, aprotinin, lactoferrin, RAP, or aprotinin, or portions thereof with LRP1B or LRP binding activity. In some embodiments, the conjugate according to the invention does not comprise PAI-1, plasminogen, pro-uPA, tissue factor inhibitor, tPA, activated α_2 -macroglobulin, α_1 -chymotrypsin, cathepsin G, lactoferrin, thyroglobulin, circumsporozite protein, saposin, gentamycin, polymixin B, pseudomonas exotoxin A, seminal vesicle secretory protein A, thrombospondin -1, β -VLDL, chylomicron remnants, IDL, Lp(a), VLDL (very low density lipoprotein), ApoB100 (apolipoprotein B 100), and Apolipoprotein E (Apo E), or portions thereof with LRP1B or LRP binding activity.

[30] In some embodiments according to the invention, the conjugate comprises PAI-1, plasminogen, pro-uPA, tissue factor inhibitor, tPA, activated α_2 -macroglobulin, α_1 -chymotrypsin, cathepsin G, lactoferrin, thyroglobulin, circumsporozite protein, saposin, gentamycin, polymixin B, pseudomonas exotoxin A, seminal vesicle secretory protein A, thrombospondin -1, β -VLDL, chylomicron remnants, IDL, Lp(a), VLDL (very low density lipoprotein), ApoB100 (apolipoprotein B 100), and Apolipoprotein E (Apo E), or portions thereof with LRP1B or LRP binding activity.

[31] In preferred embodiments of the invention, the p97 or LRP1 or LRP1B ligand or modulator of human or mammalian origin. In other embodiments, the ligand is the native compound from the human or mammal. In other embodiments, the ligand is substantially homologous (at least 60% identical in amino acid sequence or atomic structure) to the native or endogenous ligand. In other embodiments, the p97 receptor is human. In other embodiments, the subject to which the conjugate, modulator, or ligand is to be administered is human.

BRIEF DESCRIPTION OF THE DRAWINGS

[32] Figure 1. Permeability coefficient (P_e) of sucrose with BBCECs. The permeability coefficient (P_e) for sucrose was calculated as previously described (Dehouck, *et al.*, *J. Neurochem.*, 58:1790-1797 (1992)) using filters coated or non-coated with BBCE cells co-

cultured with astrocytes. Briefly, coated or non-coated filters with BBCE cells were transferred to 6-well plates containing 2 ml of Ringer-Hepes per well (basolateral compartment) for 2 hrs at 37°C. In each apical chamber, the culture medium was replaced by 1 ml Ringer-Hepes containing labeled [¹⁴C]-sucrose. At different times, inserts were placed into another well. At the end of the experiments, amounts of the radiotracers in the basolateral compartment were measured in a liquid scintillation counter. The results were plotted as the sucrose clearance (μl) as a function of time (min). The permeability of sucrose with non-coated filters with BBCE cells (PSf) and with coated filters with BBCE cells (PSt) were obtained. The results were plotted as the clearance of [¹⁴C]-sucrose (μl) as a function of time (min). PS = permeability x surface area of a filter of the coculture; PSt = permeability of the filter and endothelial monolayer; PSf = permeability of a filter coated with collagen and astrocytes plated on the bottom side of the filter. The permeability coefficient (Pe) of the endothelial monolayer was calculated as:

$$1) \quad \text{Clearance } (\mu\text{l}) = \frac{[C]_A \times V_A}{[C]_L} \quad \begin{array}{l} [C]_A = \text{Abluminal tracer concentration} \\ V_A = \text{Volume of abluminal chamber} \\ [C]_L = \text{Luminal tracer concentration} \end{array}$$

$$2) \quad 1/Pe = (1/PSt - 1/PSf) / \text{filter area } (4.2 \text{ cm}^2)$$

[33] Figure 2. Effect of p97 (1 μM) on sucrose permeability with BBCECs that were preincubated for 2 hrs at 37°C. The effect of p97 at 1 μM on sucrose permeability of the *in vitro* BBB model was tested. The permeability coefficient (Pe) for sucrose was calculated as previously described (Dehouck, *et al.*, *J. Neurochem.*, 58:1790-179 (1992)) using filters coated or non-coated with BBCE cells. Briefly, coated or non-coated filters with BBCE cells were transferred to 6-well plates containing 2 ml of Ringer/Hepes per well (basolateral compartment) for 2 hrs at 37°C. In each apical (*i.e.*, luminal) chamber, the culture medium was replaced by 1 ml Ringer/Hepes containing labeled [¹⁴C]-sucrose. At different times, inserts were placed into another well. At the end of the experiments, amounts of the radiotracers in the basolateral (*i.e.*, abluminal) compartment were measured in a liquid scintillation counter. The permeability coefficient (Pe) was calculated as described in Figure 1.

[34] Figure 3. Binding of p97 on bovine brain capillary endothelial cell monolayers. Binding p97 was performed with BBCECs that were pre-incubated 2 hrs in Ringer-Hepes to avoid any interference from the astrocytes. For the binding experiments, cells were incubated

for 2 h at 4°C in Ringer/Hepes in the presence of [¹²⁵I]-p97 (25 nM) and 7.5 micromolar concentrations of cold-p97, transferrin or lactoferrin, respectively. At the end of the incubation, the filters were gently washed at 4°C three times with 4 ml of cold-PBS. Then the associated radioactivity of endothelial cells was determined by removing the membrane of the culture insert and counting it in a gamma counter.

[35] Figure 4. Binding of p97 on rat brain endothelial cells. Binding p97 was performed with RBE4 that were pre-incubated 2 hrs in Ringer-Hepes. For the binding experiments, cells in 24-well microplates were incubated for 2 h at 4°C in Ringer/Hepes in the presence of [¹²⁵I]-p97 (25 nM) and 10 micromolar concentrations of cold-p97, transferrin or lactoferrin, respectively. At the end of the incubation, the cells were gently washed at 4°C three times with 4 ml of cold-PBS. RBE4 cells were then lysed with Triton X-100 and the associated radioactivity of with cell lysates was determined.

[36] Figure 5. Accumulation of p97 in human brain capillaries. Accumulation of [¹²⁵I]-p97 was measured at 37°C for 1 h in isolated human brain capillaries (100 µg/assay). The incubation medium contained [¹²⁵I]-p97 and a final concentration of 100 nM p97 in Ringer/Hepes solution. The accumulation of [¹²⁵I]-p97 was performed in the presence or absence of 5 µM cold-p97, holo-transferrin or lactoferrin. After incubation, the accumulation was stopped by addition of 1 ml-cold stop solution (150 mM NaCl, 0.1% BSA and 5 mM Hepes, pH 7.5). The suspension was filtered under vacuum through a 0.45µM pore size Millipore filter. The filter was rinsed with 8 ml of stop solution, and the radioactivity was counted. Nonspecific binding of the radioactivity to the capillaries was determined by the addition of the ice-cold stop solution to the capillaries before adding the incubation medium. This value was subtracted from accumulation values obtained following an 1 h incubation. The results were expressed as ng of [¹²⁵I]-p97 accumulated per µg of brain capillaries.

[37] Figure 6 is a repeat experiment, performed identically to the experiment of Figure 5, except this time with the additional competition assay for β-amyloid peptide. Evidently, the β-amyloid peptide 1-40 competes with p97 for receptor binding, along with lactoferrin, but not with transferrin.

[38] Figure 7 illustrates the results of experiments where the ligands p97, Lf and Tf were heated or not heated prior to the binding study. In all cases, binding experiments were conducted in the transwell apparatus as described previously, with the exception that binding was conducted at either 4°C or at 37°C. For the 37°C trial, a separate experiment was

conducted where the ligand was boiled for 30 mins then rapidly cooled prior to administration on the transwell plates.

[39] Figure 8 demonstrates that p97 transcytosis was significantly higher at 37°C than at 4°C. This result demonstrates that p97 is actively transported in an energy-dependent process across this blood-brain barrier model in a temperature-dependent fashion, presumably by receptor mediated uptake.

[40] Figure 9 confirms that transcytosis of p97 is also a saturable phenomenon, thus further implicating a specific MTF-receptor protein in this model of the blood-brain barrier. These experiments were conducted as previously described. Measurements of the amount of transcytosis were made at the time points indicated.

[41] Figure 10 assesses the ability of several agents to interfere with p97 transcytosis. Figure 10a, transcytosis of I125-P97 was compared in the presence of cold p97 (5 micromolar), Lf (5 micromolar), and Tf (5 micromolar). Figure 10b, β -amyloid protein (5 micromolar) also failed to slow or reduce transcytosis of labelled p97. Figure 10c, RAP, a known poly-peptide inhibitor of the LDL-Receptor family was applied to the cells (25 micrograms/ml). RAP significantly inhibited the transcytosis of p97, thus directly implicating the LDL-receptor family, especially LRP1 as the MTF-R.

[42] Figure 11 examines the accumulation and transcytosis of p97. Figure 11a, of [125 I]-p97, [125 I]-BSA and [125 I]-holo-transferrin one hour after i.v. injection. The radioactivity in brain was compared between the three compounds. (n=3); Figure 11b, Accumulation of p97 and transferrin into the mouse brain parenchyma. In situ brain perfusion was performed with human [125 I]-p97 or [125 I]-holo-transferrin at a final concentration of 10 nM via a catheter inserted in the right common carotid artery. The volume of distribution (V_D) of [125 I]-proteins were obtained in the whole brain homogenate (white bars), in brain capillaries (solid bars) and in brain parenchyma (hatched bars) after isolation of the right hemisphere and capillary depletion. Results were obtained for p97 (n=10) and for holo-transferrin (n=6); Figure 11c, Transcytosis of p97 across BBCEC monolayers. Transcytosis experiments were performed 37 °C (open circles) and 4 °C (closed circles) with BBCEC monolayers. [125 I]-p97 (1mg/ml) was added to the upper side of the cell-covered filter. One representative experiment is shown (n=4); Figure 11d, Preferential transport of p97 across the BBCEC monolayers. Apical-to-basal and basal-to-apical transport of [125 I]-p97 (25 nM) was measured

for 2 hrs at 37°C. At the end of the experiment, [¹²⁵I]-p97 was assessed in the lower or upper chambers of each well by TCA precipitation (n=4).

[43] Figure 12 illustrates the Binding of p97 to BBCE cells. Figure 12a, p97 binding experiments were performed with BBCECs that were either pre-incubated in Ringer-Hepes solution or pre-treated with saponin. BBCECs were then incubated for 2 hrs at 4 °C with [¹²⁵I]-p97 (25 nM). At the end of the incubation, the filters were gently washed with cold PBS and then the radioactivity associated with the ECs was quantified; Figure 12b, The binding of [¹²⁵I]-p97 was also performed with increasing concentrations of unlabelled p97 following saponin treatment. The results were expressed as the percentage of the [¹²⁵I]-p97 binding measured in the absence of unlabelled p97; Figure 12c, The results were also transformed with a Scatchard plot and expressed as the ratio of bound p97/free p97 as a function of the bound p97 (n=5).

[44] Figure 13 illustrates the transcytosis and accumulation of p97 and transferrin in BBCEC monolayers. Figure 13a, Transcytosis experiments were performed at 37 °C (solid bars) or 4 °C (white bars). [¹²⁵I]-p97 or bovine [¹²⁵I]-holo-transferrin (1 mg/ml) was added to the upper side of the cell-covered filter. At the end of the experiment, radiolabelled proteins were measured in the lower chamber of each well by TCA precipitation (n=4). Controls were also performed at 37 °C with denatured [¹²⁵I]-p97 or bovine [¹²⁵I]-holo-transferrin boiled for 30 min (grey bars) (n=2); Figure 13b, Biospecific interaction analysis was performed with native or boiled p97 for the indicated times. MAb L235 (5 µg) was immobilized on a sensor chip (SM5) using standard procedures incorporating NHS, EDC and ethanolamine. Native and boiled p97 (5 to 30 min) diluted at 1 mg/ml in Ringer/Hepes was cooled and injected into the BIAcore. The surface plasmon resonance response obtained for native p97 and boiled p97 was plotted (in relative units (RU)) as a function of time; Figure 13c, The accumulation of both proteins into BBCEC cells were also measured. Briefly, after incubation at 37 °C (solid bars) or 4 °C (white bars) with either [¹²⁵I]-protein, cells were washed four times with cold PBS. Accumulation of both denatured proteins (grey bars) was also measured at 37 °C. Filters were then removed, and the radioactivity associated with the cells was quantified (n=3).

[45] Figure 14 illustrates the stability of p97 and integrity of the BBCEC monolayers following p97 transcytosis. Figure 14a, Transcytosis experiments were performed at 37 °C and 4 °C by adding p97 (1 mg/ml) to the upper compartment. At the end of the experiment,

50 μ l from each lower chamber was used for SDS-polyacrylamide gel electrophoresis. After electrophoresis, the gels were stained with Coomassie Blue. A standard curve was also made with known amounts of recombinant p97 (0-2 μ g); Figure 14b, The gels were dried and scanned to quantify the amount of intact p97 that crossed the BBCEC monolayers at 37 °C and 4 °C. (n=3); Figure 14c, Effect of p97 on sucrose permeability of BBCE cell monolayers co-cultured with astrocytes. The passage of [14 C]-sucrose was measured with filters (a) or with filters coated with BBCE cells in the absence (open circles) or in the presence of p97 (up to 1 mg/ml (closed circles)). One representative experiment is shown. The results were plotted as the sucrose clearance (μ l) as a function of time (min); Figure 14d, The sucrose permeability coefficient (Pe) was determined in the presence (+p97) or in the absence (-p97) of p97, and was calculated as described in the Examples (n=3).

[46] Figure 15 shows the inhibition of [125 I]-p97 transcytosis in BBCEC monolayers. Figure 15a, Transport of [125 I]-p97 (25 nM) from the apical to the basolateral side of ECs was measured in the absence (open circles) or in the presence (closed circles) of a 200-fold molar excess of unlabelled p97; Figure 15b, The effects of a 200-fold molar excess of either human or bovine transferrin (Tf) and p97 were also evaluated on [125 I]-p97 transcytosis across BBCEC monolayers (n=5); Figure 15c, Transcytosis of [125 I]-p97 (25 nM) was also measured in the presence of mouse IgGs (open circles) or mAb OX-26 (closed circles) at a concentration of 5 μ g/ml (n=3)

[47] Figure 16 illustrates the identification of a p97 receptor according to ligand specificity. Figure 16a, Uptake of [125 I]-p97 into human brain capillaries. The uptake of [125 I]-p97 at 100 nM (control) into isolated human brain capillaries was measured for 1 h at 37 °C in the presence of a 50-fold molar excess of unlabelled p97, human holo-transferrin or human lactoferrin. (* P < 0.5 Student's t-test (n=5); Figure 16b, Effect of RAP on [125 I]-p97 transcytosis across BBCEC monolayers. Apical-to-basal transport of [125 I]-p97 was measured in the presence (closed circles) or absence (open circles) of RAP (25 μ g/ml) (n=5); Figure 16c, Inhibition of bovine [125 I]-lactoferrin transport by p97. Transcytosis of bovine [125 I]-lactoferrin (50 nM) was measured in the presence (closed circles) or absence (open circles) of unlabelled p97 (5 μ M) at 37 °C (n=3).

[48] Figure 17. Examination of the role of LRP on p97 transcytosis. Figure 17A. Apical-to-basal transport of [125 I]-p97 was measured for 2 h at 37°C in the absence (Control) or presence of RAP (0.6 μ M), aprotinin (5 μ M) or BSA (5 μ M). Results represent means \pm SD (n=5 for RAP; n=3 for aprotinin and BSA). [125 I]-p97 was assessed in the lower chamber of

each well by TCA precipitation. Figure 17B. Inhibition of bovine [125 I]-lactoferrin transport by p97. Transcytosis of bovine [125 I]-lactoferrin (50 nM) was measured in the presence or absence of unlabelled p97 (5 μ M) at 37°C for 2 hrs. Bovine [125 I]-lactoferrin was assessed in the lower or upper chambers of each well by TCA precipitation. Results represent means \pm SD (n=6). Some ligands for LRP and megalin are presented in Figure 17C.

[49] Figure 18 illustrates the time-course of the uptake of p97 in BBCE and accumulation in early endosome.

[50] Figure 19 is a schematic of how the p97 transport rate across the blood brain barrier of the model is determined.

[51] Figure 20 presents the results of measuring the rate of internalization and transcytosis of p97 in the blood brain barrier model.

[52] Figure 21 depicts the transcellular co-localization of p97 and clathrin during transcytosis.

[53] Figure 22 is a schematic drawing of two of several pathways possible following endocytosis of a p97 compound or conjugate: transcytosis and lysosomal delivery.

[54] Figure 23 is a schematic drawing of the LRP receptor associated with a cell membrane and a number of the ligands of such LRP receptors.

[55] Figure 24. Uptake of p97 in rat astrocytes and astrocytoma cells. Cells were grown in monolayer in six-multiwell microplates at 37°C under 5% CO₂. Uptake of [125 I]-p97 was measured at 37°C for 2 h in astrocytes and astrocytomas. The incubation medium contained [125 I]-p97 and a final concentration of 50 nM p97 in Ringer/Hepes solution. After incubation, the cell monolayer was washed three times with cold Ringer/Hepes solution. Triton X-100 0.1% was added and the [125 I]-p97 uptake was assessed in the Triton X-100 soluble fraction by TCA precipitation. Results represent means \pm SD (n=3, Astro; n=6, CTX and RG2; n=9, C6).

[56] Figure 25. Immunodetection of LRP/LRP 1B. Cells lysates (25 (g) were subjected to SDS-PAGE under non-reducing conditions and electroblotted onto PVDF membranes. LRP/LRP1B was immunodetected using a rabbit polyclonal antibody raised against the low density lipoprotein receptor-related protein as previously described (Bu et al., J. Biol. Chem. 17:13002-13009, 1993). (n=1)

[57] Figure 26. Immunodetection of megalin. Cells lysates were subjected to SDS-PAGE under non-reducing conditions and electroblotted onto PVDF membranes. Megalin was immunodetected using a mouse monoclonal antibody diluted 1/2500 in TBS-Tween 0.1%, 3% BSA and 0.01% NaN₃. (n=1)

5 [58] Figure 27. mRNA extraction and RT-PCR of LRP 1B. For RNA extraction, cells were grown in 75 cm² plastic tissue culture flasks at 37°C under 5% CO₂ with optimal culture medium to 80-90% confluence. Total RNA from each cell line was extracted as described in the Examples. The amplified PCR products were electrophoresed on a 2% agarose gel and visualized under ultraviolet light. (n=1).

10 [59] Figure 28 presents some results of experiments correlating p97 uptake with LRP1B expression.

[60] Figure 29. LRP/LRP1B migrates as a high molecular weight dissociable complex in presence of p97. U-87 cells were grown in monolayer in six-multiwell microplates at 37°C under 5% CO₂. The cell monolayer were incubated at 4°C with p97 or RAP in Ringer/Hepes solution. After incubation, cell monolayer was washed 3 times with cold- Ringer/Hepes solution and proteins were cross-linked with 1mM DSS. After, cells were lysed and equal quantities of protein (25 µg) were subjected to SDS-PAGE under non-reducing conditons and electroblotted onto PVDF membranes. LRP/LRP1B was immunodetected as described in Examples using a mouse monoclonal antibody raised against human LRP α-subunit (clone 8G1 from Research Diagnostics inc.) at a 1/500 dilution in TBS-Tween 0.1%, 3% BSA and 0.01% NaN₃. (n=2).

[61] Figure 30. Reducing condition induces release of p97 from high molecular weigh complex. U-87 cells were grown in monolayer in six-multiwell microplates at 37°C under 5% CO₂. The cell monolayer were incubated at 4°C with [¹²⁵I]-p97 in Ringer/Hepes solution. After incubation, cell monolayer was washed with cold-Ringer/Hepes solution and [¹²⁵I]-p97 cross-linked was performed with 1 mM DTSP according to the manufacturer's protocol. After, cells were lysed and proteins were subjected to SDS-PAGE under reducing conditions or not. Autoradiography of [¹²⁵I]-p97 was performed following gel fixation and drying. (n=2)

30 [62] Figure 31. p97 migrates as a high molecular weight protein complex. The ligand binding was performed as described in the Examples. Concerning human brain capillaries, the ligand binding was performed on 100µg capillaries with the same protocol as cell

monolayer. After ligand binding, cells were lysed with lysis buffer at 4°C for 30 min. Equal quantities of protein were subjected to SDS-PAGE under non-reducing conditions.

Autoradiography of [¹²⁵I]-p97 was performed following gel fixation and drying. (n=2)

[63] Figure 32. Expression of members of LDL receptor family (RT-PCR). RNA from isolated human capillaries and U87 were performed as described in the Examples. The amplified PCR products were electrophoresed on a 2% agarose gel and visualized under ultraviolet light. (n=2)

[64] Figure 34. Effect of p97 and RAP treatments in U-87 cells. A. Cells (2 x 10⁵) were plated onto six-multiwell microplates and grown at 37°C under 5% CO₂ with optimal culture medium supplemented with serum for 4 days. Treatment was performed in serum free medium for 72 hours. RNA isolation and RT-PCR were performed as described in the Examples. B. LRP/LRP1B was immunodetected in cells lysates as described in the Examples. Mouse monoclonal antibody raised against human LRP α₂-subunit (clone 8G1 from Research Diagnostics inc.) was used at a 1/500 dilution in TBS-Tween 0.1%, 3% BSA and 0.01% NaN₃. (N=2 for p97 treatment), (n=1 for RAP treatment)

[65] Figure 35. Effect of p97 and RAP treatments in U-87 cells (RT-PCR). Cells (2 x 10⁵) were plated onto six-multiwell microplates and grown at 37°C under 5% CO₂ with optimal culture medium supplemented with serum for 4 days. Treatment was performed in serum free medium for 72 hours. RNA isolation and RT-PCR were performed as described in the Examples. The amplified PCR products were electrophoresed on a 2% agarose gel and visualized under ultraviolet light. (n=2 for p97), (n=1 for RAP)

[66] Figure 36. Effect of p97 and RAP treatments in U-87 cells (RT-PCR) on endogenous melanotransferrin. Cells (2 x 10⁵) were plated onto six-multiwell microplates and grown at 37°C under 5% CO₂ with optimal culture medium supplemented with serum for 4 days. Treatment with p97 and RAP was performed in serum free medium for 72 hours. RNA isolation and RT-PCR were performed as described in the Examples. The amplified PCR products were electrophoresed on a 2% agarose gel and visualized under ultraviolet light. (n=1).

[67] Figure 37. Effect of p97 and RAP treatments in U-87 cells. Figure 37a Agarose gel electrophoresis of PCR products. The amplified PCR products were electrophoresed on a 2% agarose gel and visualized under ultraviolet light. (n=1).

[68] Figure 38. Effect of p97 and RAP treatments in U-87 cells. The amplified PCR products were electrophoresed on a 2% agarose gel and visualized under ultraviolet light. (n=1). Quantification was by densitometry of p97 and RAP treatments on the RNA levels of LRP1B, LRP, LDL-R, cubilin, endogenous p97 and megalin. Results are expressed as ratios of RNA levels between control and treated U-87 cells.

[69] Figure 39 show the expression of members of the LDL receptor family in MG1391. RT-PCR. RT-PCR was performed for members of the LDL-R family: LRP, LRP1B, megalin, LDL, VLDL, LRP8, LR11, LRP5 and cubulin. DNA (cDNA) synthesis was performed with 1 µg of total RNA using a cDNA one step synthesis kit (Invitrogen,USA) following the manufacturer's protocol. (1x of reaction mix, RNA 1µg, 0.2µM of both primers, 1µl of RT/Platinum Tag mix). The cDNA generated was amplified using primers produced with MacVector 7.0 (Oxford molecular Ltd, Oxford, UK). All the subsequent assays were then performed under conditions that produced amplifications of cDNA within a linear range. RT inverse-transcription was performed at 50°C for 30 min. PCR amplification for 35 or 40 cycles for all was performed as follows: denaturation at 94°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 1 min. Finalisation stage was performed at 72°C for 5 min. Tubes containing all the ingredients except templates were included in all runs and served as negative controls. The amplified PCR products were electrophoresed on a 1% agarose gel in TAE (40 mM Tris, 360 mM acetic acid, 1 mM EDTA, 12.5 fM Ethidium bromide) and were visualized under ultraviolet light followed by densitometric analysis.

[70] Figure 40 shows the effect of p97 treatment on the expression of LRP1B, LRP, LDL-R, and LRP5 in MG1391 cells using the above methods.

[71] Figure 41 shows the effect of p97 on the expression of LRP1B, LRP, LDL-R, and LRP5 in MG1391 cells using the above methods.

[72] Figure 42 shows the expression of LDL-receptor family members in human endothelial cells.

[73] Figure 43 shows the expression of LDL receptor family members in BBCE cells cultured in the presence and absence of astrocytes.

DETAILED DESCRIPTION OF THE INVENTION

A. Overview

5 [74] The present invention provides, *inter alia*, novel compounds, pharmaceutical compositions and methods for modulating the activity of the melanotransferrin receptor and for modulating the transcytosis, endocytosis, and blood brain barrier transport of active agents conjugated to melanotransferrin ("MTf" or "p97") or other ligands of LRP1, and more particularly, LRP1B. It also provides screening methods for identifying such
10 compounds, compositions, and methods. The invention is based on the discovery, disclosed here for the first time, that melanotransferrin binding to brain capillary endothelial cells (BCECs) is competitively inhibited by lactoferrin (Lf), RAP, and β -amyloid protein, but not significantly by transferrin (Tf) or bovine serum albumin (BSA). This binding spectra establishes the p97 receptor is a member of the family of LRP family. This assignment is
15 consistent with the characterization herein of the mechanism for p97 transcytosis and endocytosis.

[75] The BCEC receptor for MTf is therefore not the transferrin receptor (Tf-R), as previously hypothesized, but an unrelated class of receptor (herein called the "MTf-R" or "p97 receptor") for which Lf is a competitive inhibitor of p97. This surprising discovery
20 provides a new pathway for the uptake of MTf into the brain; and simultaneously identifies a new role for receptors of lactoferrin and/or β -amyloid proteins in BCECs, namely MTf binding and uptake.

[76] The invention also relates to the unexpected discovery that LRP1B is a major p97 receptor involved in the transcytosis and endocytosis of p97. Applicants have, for instance,
25 found that p97 binds to the LRP1B receptor to form a high molecule weight complex which is dissociable and subject to competitive inhibition. Applicants have also found that the transcytosis and uptake of p97 in cells is associated with their expression of the LRP1B gene and that LRP1B is induced by treatment with p97.

[77] In addition, it has been discovered that, in contrast to several other tumors, the LRP1B
30 gene is upregulated in astrocytomas. In a preferred embodiment, therefore, p97 and ligands binding LRP1B are conjugated to chemotherapeutic agents active against such cells.

[78] In a preferred embodiment of the invention, MTf-R biological activity is modulated to influence blood-brain barrier transport of compounds conjugated to p97, and in a further preferred embodiment, MTf-R biological activity is modulated to influence the transport of melanotransferrin conjugated therapeutic agents (MTf-TA) into the brain.

5 [79] In a further preferred embodiment, modulation of delivery across the blood-brain barrier is achieved by modulating the activity and/or expression of MTf-Rs on the serum face (*i.e.*, the apical side, or the “inside” of the blood vessel) of brain capillary endothelial cells. The present invention therefore provides, *inter alia*, methods of modulating MTf-TA uptake into the brain, compositions useful for modulating MTf-TA uptake into the brain, and
10 screening assays and methods for identifying modulators of MTf-TA uptake into the brain. The screening assays and compounds identified using such screening assays can also be used for modulating the expression and/or activity of Lf-Rs in general, and thus for treating the diseases and disorders associated with the expression and/or activity of Lf-Rs, such as those disclosed hereinbelow.

15 [80] The blood-brain barrier (BBB) performs a neuroprotective function by tightly controlling access to the brain; consequently it also impedes access of pharmacological agents to cerebral tissues, necessitating the use of vectors for their transit. We have discovered that recombinant human melanotransferrin is highly transported into the brain by using animal models and a well-defined in vitro model of the BBB. Transcytosis of p97 is at
20 least 14-fold higher than that of holo-transferrin, with no apparent intraendothelial degradation. The transport of p97 is not due to changes in endothelial barrier integrity but to receptor-mediated endocytosis. We have also discovered a member of the low-density lipoprotein receptor protein family, likely LRP, is involved in p97 transendothelial transport. The brain accumulation, high rate of p97 transcytosis and its very low level in the blood
25 (100,000-fold lower than transferrin) indicate that p97 is particularly and advantageous carrier as a delivery system to target drugs directly to the brain.

[81] Blood-brain barrier (BBB) permeability is frequently a rate-limiting factor for the penetration of drugs or peptides into the central nervous system (CNS) (see Pardridge, W.M. *J. Neurovirol.* 5: 556-569 (1999); Bickel, U., Yoshikawa, T. & Pardridge, W.M. *Adv. Drug*
30 *Deliv. Rev.* 46: 247-279 (2001)). The brain is shielded against potentially toxic substances by the BBB, which is formed by brain capillary endothelial cells that are closely sealed by tight junctions. In addition, brain capillaries possess few fenestrae and few endocytic vesicles, compared to the capillaries of other organs (see Pardridge, W.M. *J. Neurovirol.* 5: 556-569

(1999)). There is little transit across the BBB of large, hydrophilic molecules aside from some specific proteins such as transferrin, lactoferrin and low-density lipoproteins, which are taken up by receptor-mediated endocytosis (see Pardridge, W.M. *J. Neurovirol.* 5: 556-569 (1999); Tsuji, A. & Tamai, I. *Adv. Drug Deliv. Rev.* 36: 277-290 (1999); Kusunohara, H. & Sugiyama, Y. *Drug Discov. Today* 6:150-156 (2001); Dehouck, B. et al. *J. Cell. Biol.* 138: 877-889 (1997); and Fillebeen, C. et al. *J. Biol. Chem.* 274: 7011-7017 (1999)).

[82] Melanotransferrin is a glycosylated protein that was first named human melanoma antigen p97 when it was found at high levels in malignant melanoma cells (see Brown, J.P., Woodbury, R.G., Hart, C.E., Hellstrom, I. & Hellstrom, K.E. *Proc. Natl. Acad. Sci. U.S.A.* 78: 539-543 (1981); and Brown, J.P. et al. *Nature* 296:171-173 (1982)). It was later renamed Mtf due to its high level of sequence homology (37-39%) with human serum transferrin, human lactoferrin and chicken transferrin. (See Brown, J.P. et al. *Nature* 296:171-173 (1982), Rose, T.M. et al. *Proc. Natl. Acad. Sci. U.S.A.* 83,1261-1265 (1986)). In contrast to transferrin and lactoferrin, no cellular receptor for p97 has been identified. It has also been shown that p97 reversibly binds iron and that it exists in two forms, one of which is bound to cell membranes by a glycosyl phosphatidylinositol anchor while the other form is both soluble and actively secreted (see Baker, E.N. et al. *FEBS Lett.* 298: 215-218 (1992); Alemany, R. et al. *J. Cell Sci.* 104: 1155-1162 (1993); and Food M.R. et al. *J. Biol. Chem.* 269: 3034-3040 (1994)). The exact physiological role of membrane-bound p97 remains to be clearly established while the function of secreted p97 is largely unexplored (see Sekyere, E. and Richardson, D.R. *FEBS Lett.* 483: 11-16, (2000)).

[83] More recently, it was reported that p97 mRNA is also widespread in normal human tissues, with the highest levels in salivary glands (see Richardson, D.R. *Eur J Biochem.* 267: 1290-1298 (2000)). In normal human brain, p97 is present in capillary endothelium whereas in brain from patients with Alzheimer's disease it is located in microglial cells associated with senile plaques (see Rothenberger, S. et al. *Brain Res.* 712: 117-121 (1996); Jefferies, W.A. et al. *Brain Res.* 712: 122-126 (1996); and Yamada, T. et al. *Brain Res.* 845: 1-5 (1999)). Serum contains very low levels of p97 (Brown, J.P., Woodbury, R.G., Hart, C.E., Hellstrom, I. & Hellstrom, K.E. *Proc. Natl. Acad. Sci. U.S.A.* 78: 539-543 (1981)). The fact that p97 levels are very low in serum while high p97 levels are reported in senile plaques shows that p97 crosses the BBB to a greater extent than do other proteins present in the serum.

[84] To investigate this hypothesis we evaluated the uptake of p97 in brain following its administration in animals and compared it to those of holo-transferrin and bovine serum albumin (BSA). We further studied and characterized p97 transcytosis using a well-established model of the BBB, consisting of bovine brain endothelial cells (BBCECs) co-cultured with rat astrocytes. (see Fillebeen, C. et al. *J. Biol. Chem.* 274: 7011-7017 (1999); (Dehouck, M.P. et al. *J. Neurochem.* 58: 1790-1797 (1992)). We also used isolated human brain capillaries for measuring p97 uptake.

[85] The results obtained with in vivo and in vitro models show a much greater passage of p97 across the BBB than holo-transferrin and further indicate the low-density lipoprotein receptor-related protein (LRP) might be involved in its passage.

[86] p97 transcytosis is 14 times higher than transferrin transcytosis. Transcytosis is mediated by a receptor as it is temperature sensitive, saturable, and p97 conformation-dependent. Transcytosis of p97 occurs without compromising the structural integrity of the blood brain barrier (BBB) and p97 is not substantially degraded.

B. Definitions

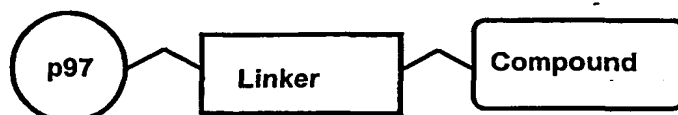
[87] "Melanotransferrin," as used herein, is sometimes referred to as "MTf" or "p97". As used in this disclosure, MTf includes membrane bound p97 (*i.e.*, p97 attached to a GPI anchor or some other anchor), secreted p97, soluble p97, cleaved p97, analogs of p97 which are equivalents of p97 (having greater than 40% homology at the peptide sequence level, including allelic variants of p97), human, mouse, chicken and/or rabbit p97, and derivatives, portions, or fragments thereof. p97 can be in the form of acidic or basic salts, or in its neutral form. In addition, individual amino acid residues can be modified, such as by oxidation or reduction. Moreover, various substitutions, deletions, or additions can be made to the amino acid or nucleic acid sequences, the net effect of which is to retain or improve upon the desired biological activity of p97. Due to code degeneracy, for example, there may be considerable variation in nucleotide sequences encoding the same amino acid sequence.

[88] "p97 fragment," as used herein, includes any portion of p97 or its biologically equivalent analogs that contains a sufficient portion of p97 to enable it to bind to the MTf-R and to be transported across the blood-brain barrier; or that otherwise retains or improves upon the desired biological activities of p97.

[89] "Melanotransferrin conjugated therapeutic agent" or, alternatively, "MTf-TA," as used herein, refers to a composition comprising p97 or a p97 fragment covalently conjugated

to another compound. The conjugation can be direct or indirect (*i.e.*, through an extended linker) so long as it is a chemical conjugation. Linkers may be as taught in U.S. Provisional Patent Application titled, "The Use of Isocyanate Linkers for the Synthesis of Hydrolyzable Active Agent Biopolymer Conjugates" with inventors Qingqi Chen, Damian Sowa, and Reinhard Gabathuler, filed on July 12, 2002 and assigned to the same assignee as the present application, and herein incorporated by reference in its entirety.

[90] The general construct of the MTf-TA of the present invention is as follows:



[91] Melanotransferrin conjugated therapeutic agents (MTf-TAs) can be used to treat many diseases including, but not limited to, neurological diseases and conditions such as Alzheimer's Disease, Parkinson's Disease, schizophrenia, epilepsy and others; neurological cancers, such as primary brain tumors including glioma, meningioma, neurinoma, pituitary adenoma, medulloblastoma, craniopharyngioma, hemangioma, epidermoid, sarcoma and intracranial metastasis from other tumor sources; and neurological infections or inflammatory conditions. Further, MTf-TAs can be used to treat non-CNS (*i.e.*, non-BBB delimited) diseases, such as cancers, diseases and conditions of non-CNS organs. Detailed descriptions of MTf-TAs and their uses are set out in U.S. Patent Application Nos. 60/226,242 and 60/226,254, the teachings of which are incorporated herein by reference.

[92] "Modulate," as used herein, refers to the ability to alter, by increase or decrease (*e.g.*, to act as an antagonist or agonist).

[93] "Melanotransferrin receptor" ("MTf-R"), as used herein, refers to any biological system that specifically or preferentially binds MTf. This term is intended to include those receptors which competitively bind Lf and/or β -amyloid protein, but excludes those receptors which are specific for Tf such as the transferrin receptor (Tf-R) (which is described at OMIM # *190010, and which is also known as TFR, TRFR and CD71). A receptor known to specifically or preferentially bind Lf is herein called a "lactotransferrin receptor" (Lf-R). Known Lf-Rs include, but are not limited to, the LDL-related receptors. A known LDL-related receptor is lipoprotein receptor-related protein/ α 2-macroglobulin receptor ("LRP1"). The term MTf-R specifically includes other receptors found on endothelial cells that specifically bind both MTf and Lf, but not Tf. In a preferred embodiment, the MTf-R is the LRP1. In a more preferred embodiment, the MTf-R is LRP1B.

[94] Members of the low density lipoprotein (LDL) receptor family include LDL-R (132 kDa); LRP/LRP1 and LRP1B (600 kDa); Megalin ((LRP2), 600 kDa); VLDL-R (130 kDa); ER-2 (LRP-8, 130 kDa); Mosaic LDL-R (LR11, 250 KDa); and other members such as LRP3, LRP6, and LRP-7. Characteristic features of the LDL-R family include cell- surface expression; extracellular ligand binding domain repeats (DxSDE); requirement of Ca⁺⁺ for ligand binding; recognition of RAP and ApoE; EGF precursor homology domain repeats (YWTD); single membrane spanning region; internalization signal in the cytoplasmic domain (FDNPXY); and receptor mediated endocytosis of various ligands.

[95] LRP refers to the low density lipoprotein receptor related protein and members of this receptor family. LRP is a large protein of 4525 amino acids (600 kDa) which is cleaved by furin to produce two subunits of 515-(α) and 85-(β) kDa that remain non-covalently bound. LRP is mainly expressed in the liver, kidney, neuron, CNS, BBB, SMC and various cultured cells.

[96] LRP ligands. A number of molecules are known to bind LRP. These molecules include, for instance, lactoferrin, RAP, lipoprotein lipase, ApoE, Factor VIII, β -amyloid precursor, α 2-macroglobulin, thrombospondin 2 MMP-2, MPP-9-TIMP-1; uPA:PAI-I:uPAR; and tPA:PAI-1:uPAR (see also Figure 23).

[97] LRP 1B is a recently discovered member of the low density lipoprotein receptor family. 600 kDa multifunctional cell surface receptor. See Liu et al., *J. Biol. Chem.* 276 (31):28889-28896 (2001). See also Liu et al., *Genomics* 69, 271-274 (2000); and Liu et al., *Cancer Res.* 60, 1961-1967 (2000). This receptor is more closely related to LRP than megalin and shares a 59% homology at cDNA level and a 52% homology at predicted amino acid level. the LRP 1B gene is expressed in the brain, thyroid and salivary gland. Known ligands for LRP 1B include RAP, tPA, PAI-1.

[98] Mouse LRP1B is accessible through GenBank Accession Nos. XM 143023 XM 130241. Human LRP1B is accessible through GenBank Accession Nos. XM 015452.

[99] "Lipoprotein receptor-related protein/ α 2-macroglobulin receptor" ("LRP1"), as used herein, refers to a multifunctional receptor. It is believed that the clustering of cysteine-rich type. A binding repeats, resembling those found in the LDL receptor, is the molecular principle for the ability to bind a variety of ligands that were previously thought to be unrelated: activated α 2-macroglobulin, apolipoprotein E, lipoprotein lipase, plasminogen activators and complexes with their inhibitor (PA and PA/PAI-1),

lipoprotein(a), pseudomonas exotoxin A, human rhinovirus, Lf and the so-called receptor associated protein (RAP). See, Meilinger, *et al.*, *FEBS Let.*, 360:70-74 (1995).

[100] LRPI is accessible through GenBank Accession No.: X 13916 and Swiss-Prot Primary Accession No.: Q07954.

- 5 [101] Alternative names for the LRP1 gene/protein include: Low-density lipoprotein receptor-related protein 1 [precursor], LRP, *Alpha*-2-macroglobulin receptor, A2MR, Apolipoprotein E receptor, APOER, CD91, LRP1 or A2MR.

[102] The screening assays and other embodiments of this invention can also employ homologs of human LRP1 (hLRP1) or LRP1B. Such homologs can be derived from other
10 organisms, specifically eukaryotes and preferably from mammals.

[103] Preferred homologs of human LRP include, but are not limited to, the following LRP proteins:

<i>H. sapiens</i> :	<u>SP:Q07954</u> - LRP1_HUMAN LOW-DENSITY LIPOPROTEIN RECEPTOR-RELATED PROTEIN 1 PRECURSOR	100 % / 4543 aa
<i>M. musculus</i> :	<u>PID:g49942</u> - AM2 receptor	97 % / 4543 aa
<i>R. norvegicus</i> :	<u>SP:P98158</u> - LRP2 RAT LOW-DENSITY LIPOPROTEIN RECEPTOR-RELATED PROTEIN 2 PRECURSOR	39 % / 4207 aa
<i>D. melanogaster</i> :	<u>SP:P98163</u> - YL DROME PUTATIVE VITELLOGENIN RECEPTOR PRECURSOR	28 % / 1550 aa
<i>C. elegans</i> :	<u>PID:g3876533</u> - predicted using Genefinder	35 % / 4205 aa
<i>S. cerevisiae</i> :	<u>PID:g557822</u> - mal5, sta1, len: 1367, CAI: 0.3, AMYH YEAST P08640 GLUCOAMYLASE S1	24 % / 1221 aa
<i>E. coli</i> :	<u>PID:g1787636</u> - putative membrane protein	25 % / 370 aa

[104] Those skilled in the art can readily identify other homologs of LRP1 or more particularly, LRP1B, suitable for use in the present invention.

- 15 [105] "Receptor for Advanced Glycation End products" ("RAGE"), as used herein, refers to a multiligand member of the immunoglobulin superfamily of cell surface molecules. RAGE was originally identified and characterized based on its ability to bind advanced glycation end products (AGEs), adducts formed by glycooxidation that accumulate in disorders such as diabetes and renal failure. Subsequent studies demonstrate that RAGE serves as a cell
20 surface receptor for amyloid- β peptide (the cleavage product of β -amyloid precursor protein (β -APP), a major component of neuritic plaques of Alzheimer's Disease). Other ligands include amphoterin and s100/calgranulin-like molecules (*see*, Hofmann, *et al.*, *Cell*,

97(7):889-901 (1999)). RAGE is readily accessible through GenBank Accession No.: M91211 and Swiss-Prot Primary Accession No.: Q15109.

[106] By “determining the functional effect” is meant assaying for a compound that modulates, *e.g.*, increases or decreases, a parameter that is indirectly or directly under the influence of the MTf-R, *e.g.*, functional, physical and chemical effects. In addition to the functional effects specifically described herein, it will be readily apparent to those of skill in the art that other functional effects can be measured by any means known to those skilled in the art, *e.g.*, changes in spectroscopic characteristics (*e.g.*, fluorescence, absorbance, refractive index), hydrodynamic (*e.g.*, shape), chromatographic, or solubility properties, changes in gene expression of MTf-Rs, and the like.

[107] “Inhibitors,” “activators” and “modulators” of MTf-Rs are used interchangeably to refer to inhibitory, activating or modulating compounds identified using *in vitro* and/or *in vivo* assays for MTf-R

[107] Samples or assays comprising MTf-Rs that are treated with a potential activator, inhibitor, or modulator are compared to control samples without the inhibitor, activator, or modulator to examine the extent of inhibition. Control samples (untreated with inhibitors) are assigned a relative MTf-R activity value of 100%. Inhibition of a MTf-R is achieved when the MTf-R activity value relative to the control is about 80%, preferably 50%, more preferably 25-0%. Activation of a MTf-R is achieved when the MTf-R activity value relative to the control (untreated with activators) is 110%, more preferably 150%, more preferably 200-500% (*i.e.*, two to five fold higher relative to the control), more preferably 1000-3000% higher.

C. Screening Assays for Modulators of Melanotransferrin Receptors (MTf-Rs)

[108] The present invention provides a screening assay employing an MTf-R, wherein compounds are tested for their ability to influence a measurable activity of the MTf-R. The MTf-R can be situated in a whole cell, a cell extract, semi-purified, purified or any other format that allows for measurement of its activity. The activity can be any activity in the expression, function or degradation of MTf-R including, for example, the amount or timing of such activities. Such activities include, for example, transcription, transcript processing, translation or transcript stability of the *MTf-R* gene sequence or mRNA transcript. Such activities include, for example, the synthesis of new MTf-R, the sub-cellular localization of MTf-R and activation of MTf-R biological activity. Such activities include, for example, the

ability of MTf-R to bind substances, adopt conformations, catalyze reactions, bind known ligands and the like. Such activities include, for example, the amount or stability of MTf-R, the processing and removal or degradation of MTf-R and the like. In preferred embodiments, the MTf-R receptor for use in screening is LRP1 or LRP1B.

5 [109] In a preferred screening assay, compounds are tested to identify modulators of a biological activity of MTf-R such as: MTf-R interactions with MTf, MTf-R interactions with Lf or other putative ligands or uptake of iron/other metal; transport of MTf across the blood-brain barrier ("BBB"), *i.e.*, in a BBB model; transport of Lf, other ligands or metals across a BBB model; and/or measurement of the rate or amount of transcription, translation or
10 expression levels of *MTf-R* genes or mRNA.

[110] The invention contemplates a variety of different screening formats. Some designs are considered low throughput and test only one or a few compounds in series or in parallel. High throughput screening assays are suitable for screening tens of thousands or hundreds of thousands of compounds in a matter of weeks or months. "In silico" screening formats
15 employ computer-aided rational design techniques to identify potential modulators of MTf-R biological activity.

[111] The test compounds of the invention may be obtained from any source, but a preferred commercial embodiment employs commercially available compound libraries of hundreds of thousands of compounds, many of which are potential therapeutic agents. These
20 compounds are tested in series or in parallel to identify modulating activity of MTf-R activity. Preferred test compounds can be identified by examining known and putative ligands of MTf-R for predicting chemical structure of inhibitors, *etc.* Other computer-aided design techniques can be employed to eliminate unsuitable candidates, such as those candidates thought to cause toxic side-effects. Those skilled in the art are familiar with the
25 combinatorial and medicinal chemistry techniques that can be used to further select test compounds and the potential therapeutic agents of the invention.

[112] The object of the screening assays is to identify modulators of MTf-R activity that are suitable for animal or human clinical trials and as therapeutic agents. As such, the screening assays identify stimulants, agonists or antagonists of MTf-R. The method of identifying
30 stimulants, agonists or antagonists of MTf-R can comprise contacting a substance suspected of being a stimulant, agonist or antagonist with MTf and the MTf-R under conditions such that MTf is capable of binding to the MTf-R; measuring the amount of MTf bound to the

MTf-R; and determining the effect of the substance by comparing the amount of MTf bound to MTf-R with an amount determined for a control. The MTf that can be used in this method includes MTf cleaved of GPI, soluble MTf, cleaved MTf or derivatives thereof, preferably recombinant MTf. In this method of the invention, the amount of MTf bound to MTf-R can be determined by measuring the amount of MTf bound to the MTf-R, the amount of unbound MTf or the amount of unbound MTf-R. MTf bound to MTf-R can be isolated by conventional isolation techniques, for example, salting out, chromatography, electrophoresis, gel filtration, fractionation, absorption, polyacrylamide gel electrophoresis, agglutination, or combinations thereof. To facilitate the measurement of MTf bound to MTf-R or of unbound MTf, or unbound MTf-R, antibodies against MTf or MTf-R can, for example, be utilized.

[113] In one embodiment, the invention also relates to a method for identifying a stimulant, agonist or antagonist of MTf-mediated iron uptake comprising: incubating a cell expressing MTf-R on its surface and a test substance (*e.g.*, compound) suspected of being a stimulant, agonist or antagonist of MTf-R in the presence of MTf bound to iron (holo-MTf) and in the absence of transferrin, measuring the amount of iron uptake into the cell and identifying a stimulant, agonist or antagonist of MTf-R mediated iron uptake by comparing the amount of iron uptake in the cell with the amount of iron uptake in a cell from a control incubation in the absence of the test substance. Iron uptake refers to the internalization of iron into the cell across the cell plasma membrane.

D. Modulating Uptake of Melanotransferrin Conjugated Therapeutic Agents (MTf-TAs) by Modulating MTf-R Activity

[114] In another embodiment, the present invention relates to a method of using compounds that modulate MTf-R biological activity to modulate the amount of uptake into the cells of melanotransferrin conjugated therapeutic agents (MTf-TAs). In particular, the invention relates to a method of increasing the uptake of an MTf-TA into the brain, the method comprising administering a modulator of MTf-R biological activity either contemporaneously or sequentially with the MTf-TA. Alternatively, the invention relates to a method to reduce uptake of an MTf-TA into the brain comprising administering a modulator of MTf-R biological activity either contemporaneously or sequentially with the MTf-TA. In a preferred embodiment, these methods employ modulators of MTf-R that are first identified using a screening assay as described hereinabove. In preferred embodiments, the MTf-R receptor for use in screening is LRP1 or LRP1B.

[115] Those skilled in the art will appreciate that increasing MTf-TA uptake and delivery across the blood-brain barrier is useful and desirable in situations such as, but not limited to, where the MTf-TA is being used to treat a neurological condition and increased amounts of delivery provide therapeutic benefit. Those skilled in the art will appreciate that decreasing
5 MTf-TA uptake and delivery across the blood-brain barrier is useful and desirable for a variety of reasons including, but not limited to, where the MTf-TA is being used for its cardio-protective effect or used in other (non-CNS) organs and side-effects of brain uptake are to be avoided.

[116] Modulators of MTf-R activity can be readily identified using a modification of the
10 transwell apparatus set out in Example I below. In the modified form, a compound is added to the luminal surface of the cells in the transwell apparatus in combination with MTf-TAs. The compound is then scored to determine if it increases or decreases the transport of the MTf-TA across the BBCECs to the abluminal side. A library of compounds can be readily screened or tested to identify pharmacologically superior modulators.

15 **E. Diseases Treatable by Lf-R Modulators**

[117] As a result of the surprising discovery that certain MTf-Rs are Lf-Rs, it has now been discovered that modulators of Lf-R biological activity can have an impact on disease processes known to be linked to MTf biological activity. As such, the following uses for modulators of Lf-Rs identified, for example, by the screening assays of the present invention
20 have now been identified.

[118] 1) Rapidly proliferating cells, such as malignant cells, have an increased requirement for iron and must possess efficient mechanisms to obtain iron. Limiting the ability of malignant cells to acquire iron provides a method of killing tumor cells or of modulating their uncontrolled cell growth.

25 [119] 2) These findings lead to the discovery that modulators of Lf-R can be used to modulate iron uptake in cells. Iron uptake in cells can be modulated by varying the amount of Lf-R on cell surfaces, or by inhibiting Lf-R binding to MTf. Accordingly, stimulants, agonists or antagonists of Lf-R can be useful in the treatment of conditions or diseases where there is a disturbance in iron metabolism. For example, such substances are useful in the
30 treatment of conditions such as hemochromatosis, neurodegenerative diseases, ischemic tissue damage, including ischemic stroke or trauma, heart disease and tumors (e.g., skin cancer).

[120] 3) MTf plays a role in the binding and uptake of other metals such as zinc and aluminum (Sekyere, E. and Richardson, D.R., *FEBS Letters*, 483:11-16 (2000) and, thus, modulation of Lf-R activity or expression plays a role in modulating or regulating Alzheimer's Disease or metaloproteins, such as zinc-dependent metaloproteins.

5 [121] 4) Conditions that involve disturbances in iron metabolism that can be treated using the compounds and methods of the invention (*i.e.*, using stimulants, agonists and antagonists of MTf-mediated iron uptake) are, for example, those involving excessive iron absorption from the diet, defects in iron uptake into cells, excessive iron uptake into cells or those requiring regular treatment by blood transfusion (*e.g.*, dyserythropoietic anemias, in particular thalassaemia disorders. Examples of such conditions include, but are not limited to, hemochromatosis, neurodegenerative diseases (*e.g.*, Alzheimer's Disease, Huntington's Disease and Parkinson's Disease), ischemic tissue damage, heart disease and tumors, inflammation and infections (*see*, Pippard, "Clinical Use of Iron Chelation," in *Iron in Immunity, Cancer and Inflammation* (M. de Sousa and J. H. Brock (Eds.), 1989, John Wiley & Sons), which is incorporated herein by reference.

10 [122] 5) Diseases which can be treated using modulators of Lf-R activity or expression include neurological diseases and conditions such as Alzheimer's Disease, Parkinson's Disease, schizophrenia, epilepsy and others, neurological cancers, such as primary brain tumors including glioma, meningioma, neurinoma, pituitary adenoma, medulloblastoma, craniopharyngioma, hemangioma, epidermoid, sarcoma and intracranial metastasis from other tumor sources, and neurological infections or inflammatory conditions. Modulators of Lf-R activity or expression can be used to treat non-CNS (*i.e.*, non-BBB delimited) diseases, such as cancers, diseases and conditions of non-CNS organs.

20 [123] Once a potential therapeutic agent has been identified from among the test compounds, those skilled in the art are familiar with the basic pharmacological steps required to develop an acceptable human or animal therapeutic agent. For example, therapeutic doses can be derived from animal studies and human clinical trials, as can suitable pharmacological carriers and excipients.

F. Therapeutic and Diagnostic Uses of MTf-R Proteins/Polypeptides

30 [124] In another embodiment of the instant invention, a method is provided for administering a MTf-R protein, or a fragment thereof, to an animal in need thereof, to effect a therapeutic or prophylactic result. *In vivo* an MTf-R protein or fragment thereof will bind to

MTf and effectively reduce the circulating concentration of MTf. This treatment is useful in diseases where increased levels of MTf are known to be implicated in disease progression, particularly, Alzheimer's Disease (*see, Kennard, et al., Nat. Med., 2:1230-1235 (1996); U.S. Patent No. 5,981,194*). As a diagnostic tool, administration of an MTf-R protein or a

5 fragment thereof, labeled with a detectable label, will identify the location or amount of MTf in the subject. Such a tool would be particularly useful for finding tumor cells that are known to express higher levels of MTf. Presumably, labeling of MTf-R with a strong *beta* emitting particle, such as certain Iodine or Yttrium isotopes, would assist in killing tumor cells to which the MTf-R would bind.

10 [125] Other therapeutical targets relate to HIV infection as a TAT receptor, activation of the NMDA receptor with respect to synaptic plasticity, Alzheimer's disease as LRP is a receptor for APoE, alpha2-macroglobulin, APP which have all been linked to this disease; and in the regulation of lipid degrading lysosomal enzymes (SAP-dependent).

G. MTf-R Antibodies

15 [126] Antibodies that specifically recognize one or more epitopes of MTf-R, or epitopes of conserved variants of MTf-R, or peptide fragments of MTf-R are also encompassed by the invention. Such antibodies include, but are not limited to, polyclonal antibodies, monoclonal antibodies (mAbs), humanized or chimeric antibodies, single chain antibodies, Fab fragments, F(ab')₂ fragments, fragments produced by a Fab expression library, anti-idiotypic (anti-Id) antibodies, and epitope-binding fragments of any of the above.

20 [127] The antibodies of the invention can be used, for example, in conjunction with compound screening assays, as described above, for the evaluation of the effect of test compounds on expression and/or activity of the MTf-R gene product. Additionally, such antibodies can be used in conjunction with gene therapy techniques to, for example, evaluate the success of transfection of normal and/or engineered MTf-R-expression. Such antibodies can be used as antagonists of MTf-R biological activity, and can additionally be used as a method for the inhibition of abnormal MTf-R activity. Thus, such antibodies can be utilized as therapeutic agents according to this invention.

25 [128] The MTf-R antibodies of the invention can also be used in diagnostic techniques to identify the location, quantity, behavior and/or the like of MTf-R in an animal.

30 [129] For the production of antibodies, various host animals may be immunized by injection with the MTf-R, a MTf-R protein or peptide (*e.g.*, one corresponding to a functional domain

of the receptor), truncated MTf-R polypeptides (MTf-R in which one or more domains has been deleted), functional equivalents of the MTf-R or mutants of the MTf-R. Such host animals include, but are not limited to, rabbits, mice, and rats, to name but a few. Various adjuvants can be used to increase the immunological response, depending on the host species including, but not limited to, Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, dinitrophenol, and potentially useful human adjuvants such as BCG (Bacille Calmette-Guerin) and *Corynebacterium parvum*. Polyclonal antibodies are heterogeneous populations of antibody molecules derived from the sera of the immunized animals.

H. MTf-R Antisense Oligonucleotides and Ribozymes

[130] The present invention also encompasses the use of antisense oligonucleotide sequences having sequences specific for hybridization to the MTf-R (e.g., LRP1 or LRP1B) mRNA or precursor RNA, *i.e.*, sequences that specifically or selectively hybridize to MTf-R or mRNA or precursor RNA. Those skilled in the art can readily identify suitable antisense oligonucleotide sequences based on the known MTf-R mRNA sequence referenced above. Those skilled in the art are aware of modifications to the chemistry of oligonucleotides that improve their clinical utility, such as phosphorothioate modifications. MTf-R antisense oligonucleotides are an example of compounds that can be used to modulate MTf-R expression and, in particular, to down-regulate such expression. Those skilled in the art are also able to design ribozymes which achieve similar objectives. Methods of delivering such nucleic acids are described below.

I. MTf-R Gene Therapy

[131] Certain embodiments of the invention employ the use of the *MTf-R* gene in a gene therapy method. The object of such intervention is to deliver a functional copy of the MTf-R gene to a cell in need thereof in order to increase the transcription and subsequent translation and expression of the MTf-R protein in the cell. Those skilled in the art are familiar with viral, non-viral (*i.e.*, lipid based), naked DNA and polymeric methods of delivering gene therapy vectors that include the *MTf-R* gene. Such methods can employ *in vivo* or *ex vivo* gene therapy techniques known to and used by those of skill in the art.

J. Diagnosis of Neurological Diseases

[132] The present invention also relates to the diagnosis of a neurological disease comprising detecting the amount or activity of MTf-R expressed at the BBB, in neural tissue or in any tissue associated with a non-CNS target organ, such as the lung, liver, kidney, spleen, *etc.* The diagnostic method employs a diagnostic agent comprising an agent specific for MTf-R (*i.e.*, an MTf-R antibody, a ligand such as MTf or Lf, or another ligand which specifically binds MTf-R) and a detectable conjugate or label (*i.e.*, radioisotopes of technetium or iodine). Those skilled in the art can identify diseases or conditions for which the determination of MTf-R amount or localization is relevant. Most preferred are diseases or conditions, such as Alzheimer's Disease, in which MTf-R is directly implicated as a result of the present invention.

K. Binding of different Ligands to LDL-R receptor family members.

[133] A large number of ligands have been found to bind to members of the LDL-R receptor family. These ligands and their receptor are indicated in Tables 1-3.

[134] Table 1. Lipoproteins and apolipoproteins

Ligands	LDL-R	LRP	Megalin	VLDL-R	ER-2	LR11
β -VLDL	Yes	Yes	Yes	Yes	Yes	Yes
Chylomicron remnants	Yes	Yes		Yes		
IDL	Yes			Yes		
Lp(a)				Yes		
VLDL	Yes			Yes	Yes	
ApoB100	Yes		Yes			
ApoE	Yes	Yes	Yes	Yes	Yes	Yes

[135] Table 2. Protease inhibitors and protease/inhibitor complexes.

Ligands	LDL-R	LRP	Megalin	VLDL-R	ER-2	LR11
PAI-1		Yes	Yes			
Plasminogen			Yes			
Pro-uPA		Yes	Yes	Yes		
Tissue factor inhibitor		Yes				
tPA		Yes	Yes			
activated α 2-macroglobulin		Yes	No			
α 1-chymotrypsin/ cathepsin G		No	Yes			

Table 3. Other ligands.

Ligands	LDL-R	LRP	Megalin	VLDL-R	ER-2	LR11
Albumin			Yes			
ApoJ/clusterin			Yes			
ApoJ/ β -amyloid		No	Yes			
β -APP		Yes		Yes		
Lactoferrin		Yes	Yes			
RAP	Yes	Yes	Yes	Yes		
Thyroglobulin			Yes		Yes	Yes
Circumsporozite protein			Yes	Yes		
Saposin		Yes				
Gentamycin		Yes	Yes			

Ligands	LDL-R	LRP	Megalin	VLDL-R	ER-2	LR11
Polymixin B		Yes	Yes			
Pseudomonas Exotoxin A		Yes	No			
Seminal Vesicle Secretory Protein A		Yes	Yes			
Thrombospondin -1		Yes		Yes		

L. Active Agents

[136] Active agents according to the invention include agents that affect any biological process. The term “drug” or “therapeutic agent” refers to an active agent that has a pharmacological activity or benefits health when administered in a therapeutically effective amount. Examples of drugs or therapeutic agents include substances that are used in the prevention, diagnosis, alleviation, treatment or cure of a disease or condition.

[137] The active agent conjugated to the p97 protein or fragment or LRP1 modulator or LRP ligand may be any molecule, as well as any binding portion or fragment thereof, that is capable of modulating a biological process in a living host. Generally, the active agent may be of any size, but is preferably a small organic molecule that is capable of binding to the target of interest. A drug moiety of the conjugate, when a small molecule, generally has a molecular weight of at least about 50 D, usually at least about 100 D, where the molecular weight may be as high as 500 D or higher, but will usually not exceed about 2000 D.

[138] The drug moiety is capable of interacting with a target in the host into which the conjugate is administered during practice of the subject methods. The target may be a number of different types of naturally occurring structures, where targets of interest include both intracellular and extracellular targets, where such targets may be proteins, phospholipids, nucleic acids and the like, where proteins are of particular interest. Specific proteinaceous targets of interest include, without limitation, enzymes, *e.g.*, kinases, phosphatases, reductases, cyclooxygenases, proteases and the like, targets comprising domains involved in protein-protein interactions, such as the SH2, SH3, PTB and PDZ domains, structural proteins, *e.g.*, actin, tubulin, *etc.*, membrane receptors, immunoglobulins,

e.g., IgE, cell adhesion receptors, such as integrins, *etc.*, ion channels, transmembrane pumps, transcription factors, signaling proteins, and the like.

[139] The active agent or drug has a hydroxy or an amino group for reacting with the isocyanate reagent or the active agent is chemically modified to introduce a hydroxy or an amino group for reacting with the isocyanate reagent.

[140] In some embodiments, the active agent or drug will also comprise a region that may be modified and/or participate in covalent linkage, preferably, without loss of the desired biological activity of the active agent. The drug moieties often comprise cyclical carbon or heterocyclic structures and/or aromatic or polyaromatic structures substituted with one or more of the above functional groups. Also of interest as drug moieties are structures found among biomolecules, proteins, enzymes, polysaccharides, and polynucleic acids, including peptides, saccharides, fatty acids, steroids, purines, pyrimidines, derivatives, structural analogs or combinations thereof.

[141] The conjugate can comprise one or more active agents linked to the same biopolymer. For example, conjugation reactions may conjugate from 1 to 5, about 5, about 1-10, about 5 to 10, about 10-20, about 20-30, or 30 or more molecules of an active agent to the biopolymer. These formulations can be employed as mixtures, or they may be purified into specific (mol:mol) formulations. Those skilled in the art are able to determine which format and which mol:mol ratio is preferred. Further, more than one type of active agent may be linked to the biopolymer where delivery of more than one type of an agent to a target site or compartment is desired. A plurality of active agent species may be attached to the same biopolymers such as adriamycin-cisplatinum conjugate compositions where the biopolymer is a p97 related protein. Thus, the conjugates may consist of a range of mol:mol ratios and incorporate more than one type of active agent. These, too, may be separated into purified mixtures or they may be employed in aggregate. Active agents include those identified U.S. Patent No. 6,372,712 which is incorporated herein by reference.

[142] Specific drugs of interest from which the drug moiety may be derived include, but are not limited to: psychopharmacological agents, such as (1) central nervous system depressants, *e.g.*, general anesthetics (barbiturates, benzodiazepines, steroids, cyclohexanone derivatives, and miscellaneous agents), sedative-hypnotics (benzodiazepines, barbiturates, piperidinediones and triones, quinazoline derivatives, carbamates, aldehydes and derivatives, amides, acyclic ureides, benzazepines and related drugs, phenothiazines, *etc.*), central

voluntary muscle tone modifying drugs (anticonvulsants, such as hydantoins, barbiturates, oxazolidinediones, succinimides, acylureides, glutarimides, benzodiazepines, secondary and tertiary alcohols, dibenzazepine derivatives, valproic acid and derivatives, GABA analogs, *etc.*), analgesics (morphine and derivatives, oripavine derivatives, morphinan derivatives, phenylpiperidines, 2,6-methane-3-benzazocaine derivatives, diphenylpropylamines and isosteres, salicylates, p-aminophenol derivatives, 5-pyrazolone derivatives, arylacetic acid derivatives, fenamates and isosteres, *etc.*) and antiemetics (anticholinergics, antihistamines, antidopaminergics, *etc.*), (2) central nervous system stimulants, *e.g.*, analeptics (respiratory stimulants, convulsant stimulants, psychomotor stimulants), narcotic antagonists (morphine derivatives, oripavine derivatives, 2,6-methane-3-benzoxacine derivatives, morphinan derivatives) nootropics, (3) psychopharmacologicals, *e.g.*, anxiolytic sedatives (benzodiazepines, propanediol carbamates) antipsychotics (phenothiazine derivatives, thioxanthine derivatives, other tricyclic compounds, butyrophenone derivatives and isosteres, diphenylbutylamine derivatives, substituted benzamides, arylpiperazine derivatives, indole derivatives, *etc.*), antidepressants (tricyclic compounds, MAO inhibitors, *etc.*), (4) respiratory tract drugs, *e.g.*, central antitussives (opium alkaloids and their derivatives); pharmacodynamic agents, such as (1) peripheral nervous system drugs, *e.g.*, local anesthetics (ester derivatives, amide derivatives), (2) drugs acting at synaptic or neuroeffector junctional sites, *e.g.*, cholinergic agents, cholinergic blocking agents, neuromuscular blocking agents, adrenergic agents, antiadrenergic agents, (3) smooth muscle active drugs, *e.g.*, spasmolytics (anticholinergics, musculotropic spasmolytics), vasodilators, smooth muscle stimulants, (4) histamines and antihistamines, *e.g.*, histamine and derivative thereof (betazole), antihistamines (H₁ -antagonists, H₂ -antagonists), histamine metabolism drugs, (5) cardiovascular drugs, *e.g.*, cardiotonics (plant extracts, butenolides, pentadienolids, alkaloids from erythrophleum species, ionophores, adrenoceptor stimulants, *etc.*), antiarrhythmic drugs, antihypertensive agents, antilipidemic agents (clofibric acid derivatives, nicotinic acid derivatives, hormones and analogs, antibiotics, salicylic acid and derivatives), antivaricose drugs, hemostyptics, (6) blood and hemopoietic system drugs, *e.g.*, antianemia drugs, blood coagulation drugs (hemostatics, anticoagulants, antithrombotics, thrombolytics, blood proteins and their fractions), (7) gastrointestinal tract drugs, *e.g.*, digestants (stomachics, choleretics), antiulcer drugs, antidiarrheal agents, (8) locally acting drugs; chemotherapeutic agents, such as (1) anti-infective agents, *e.g.*, ectoparasitocides (chlorinated hydrocarbons, pyrethins, sulfurated compounds), anthelmintics, antiprotozoal agents, antimalarial agents, antiamebic agents, antileishmanial drugs, antitrichomonal agents, antitrypanosomal agents,

sulfonamides, antimycobacterial drugs, antiviral chemotherapeutics, *etc.*, and (2) cytostatics, *i.e.*, antineoplastic agents or cytotoxic drugs, such as alkylating agents, *e.g.*, Mechlorethamine hydrochloride (Nitrogen Mustard, Mustargen, HN2), Cyclophosphamide (Cytovan, Endoxana), Ifosfamide (IFEX), Chlorambucil (Leukeran), Melphalan (Phenylalanine

- 5 Mustard, L-sarcolysin, Alkeran, L-PAM), Busulfan (Myleran), Thiotepea (Triethylenethiophosphoramide), Carmustine (BiCNU, BCNU), Lomustine (CeeNU, CCNU), Streptozocin (Zanosar) and the like; plant alkaloids, *e.g.*, Vincristine (Oncovin), Vinblastine (Velban, Velbe), Paclitaxel (Taxol), and the like; antimetabolites, *e.g.*, Methotrexate (MTX), Mercaptopurine (Purinethol, 6-MP), Thioguanine (6-TG), Fluorouracil (5-FU), Cytarabine
 10 (Cytosar-U, Ara-C), Azacitidine (Mylosar, 5-AZA) and the like; antibiotics, *e.g.*, Dactinomycin (Actinomycin D, Cosmegen), Doxorubicin (Adriamycin), Daunorubicin (duanomycin, Cerubidine), Idarubicin (Idamycin), Bleomycin (Blenoxane), Picamycin (Mithramycin, Mithracin), Mitomycin (Mutamycin) and the like, and other anticellular proliferative agents, *e.g.*, Hydroxyurea (Hydrea), Procarbazine (Mutalane), Dacarbazine
 15 (DTIC-Dome), Cisplatin (Platinol) Carboplatin (Paraplatin), Asparaginase (Elspar) Etoposide (VePesid, VP-16-213), Amsarcrine (AMSA, m-AMSA), Mitotane (Lysodren), Mitoxantrone (Novatrone), and the like;

- [143] Antibiotics, such as: aminoglycosides, *e.g.*, amikacin, apramycin, arbekacin, bambermycins, butirosin, dibekacin, dihydrostreptomycin, fortimicin, gentamicin,
 20 isepamicin, kanamycin, micromycin, neomycin, netilmicin, paromycin, ribostamycin, sisomicin, spectinomycin, streptomycin, tobramycin, trospectomycin; amphenicols, *e.g.*, azidamfenicol, chloramphenicol, florfenicol, and theimaphenicol; ansamycins, *e.g.*, rifamide, rifampin, rifamycin, rifapentine, rifaximin; beta.-lactams, *e.g.*, carbacephems, carbapenems, cephalosporins, cephamycins, monobactams, oxaphems, penicillins; lincosamides, *e.g.*,
 25 clinamycin, lincomycin; macrolides, *e.g.*, clarithromycin, dirthromycin, erythromycin, *etc.*; polypeptides, *e.g.*, amphomycin, bacitracin, capreomycin, *etc.*; tetracyclines, *e.g.*, apicycline, chlortetracycline, clomocycline, *etc.*; synthetic antibacterial agents, such as 2,4-diaminopyrimidines, nitrofurans, quinolones and analogs thereof, sulfonamides, sulfones;

- [144] Antifungal agents, such as: polyenes, *e.g.*, amphotericin B, candicidin, dermostatin,
 30 filipin, fungichromin, hachimycin, hamycin, lucensomycin, mepartricin, natamycin, nystatin, pecilocin, perimycin; synthetic antifungals, such as allylamines, *e.g.*, butenafine, naftifine, terbinafine; imidazoles, *e.g.*, bifonazole, butoconazole, chlordanol, chlormidazole, *etc.*, thiocarbamates, *e.g.*, tolclate, triazoles, *e.g.*, fluconazole, itraconazole, terconazole;

[145] Anthelmintics, such as: arecoline, aspidin, aspidinol, dichlorophene, embelin, kosin, naphthalene, niclosamide, pelletierine, quinacrine, alantolactone, amocarzone, amoscanate, ascaridole, bethovenium, bitoscanate, carbon tetrachloride, carvacrol, cyclobendazole, diethylcarbamazine, *etc.*;

5 [146] Antimalarials, such as: achedapsone, amodiaquin, arteether, artemether, artemisinin, artesunate, atovaquone, bebeerine, berberine, chirata, chlorguanide, chloroquine, chlorprogaunil, cinchona, cinchonidine, cinchonine, cycloguanil, gentiopicroin, halofantrine, hydroxychloroquine, mefloquine hydrochloride, 3-methylarsacetin, pamaquine, plasmocid, primaquine, pyrimethamine, quinacrine, quinidine, quinine, quinocide, quinoline, dibasic
10 sodium arsenate;

[147] Antiprotozoan agents, such as: acranil, tinidazole, ipronidazole, ethylstibamine, pentamidin, acetarsone, aminitroazole, anisomycin, nifuratel, tinidazole, benzidazole, suramin, and the like.

[148] Drug compounds of interest from which drug moieties may be derived are also listed
15 in: Goodman & Gilman's, The Pharmacological Basis of Therapeutics (9th Ed) (Goodman et al. eds) (McGraw-Hill) (1996); and 1999 Physician's Desk Reference (1998).

[149] Specific compounds of interest also include, but are not limited to:

antineoplastic agents, as disclosed in U.S. Pat. Nos. 5,880,161, 5,877,206, 5,786,344, 5,760,041, 5,753,668, 5,698,529, 5,684,004, 5,665,715, 5,654,484, 5,624,924, 5,618,813,
20 5,610,292, 5,597,831, 5,530,026, 5,525,633, 5,525,606, 5,512,678, 5,508,277, 5,463,181, 5,409,893, 5,358,952, 5,318,965, 5,223,503, 5,214,068, 5,196,424, 5,109,024, 5,106,996, 5,101,072, 5,077,404, 5,071,848, 5,066,493, 5,019,390, 4,996,229, 4,996,206, 4,970,318, 4,968,800, 4,962,114, 4,927,828, 4,892,887, 4,889,859, 4,886,790, 4,882,334, 4,882,333, 4,871,746, 4,863,955, 4,849,563, 4,845,216, 4,833,145, 4,824,955, 4,785,085, 4,684,747,
25 4,618,685, 4,611,066, 4,550,187, 4,550,186, 4,544,501, 4,541,956, 4,532,327, 4,490,540, 4,399,283, 4,391,982, 4,383,994, 4,294,763, 4,283,394, 4,246,411, 4,214,089, 4,150,231, 4,147,798, 4,056,673, 4,029,661, 4,012,448;

[150] psychopharmacological/psychotropic agents, as disclosed in U.S. Pat. Nos. 5,192,799, 5,036,070, 4,778,800, 4,753,951, 4,590,180, 4,690,930, 4,645,773, 4,427,694, 4,424,202,
30 4,440,781, 5,686,482, 5,478,828, 5,461,062, 5,387,593, 5,387,586, 5,256,664, 5,192,799, 5,120,733, 5,036,070, 4,977,167, 4,904,663, 4,788,188, 4,778,800, 4,753,951, 4,690,930, 4,645,773, 4,631,285, 4,617,314, 4,613,600, 4,590,180, 4,560,684, 4,548,938, 4,529,727,

4,459,306, 4,443,451, 4,440,781, 4,427,694, 4,424,202, 4,397,853, 4,358,451, 4,324,787,
4,314,081, 4,313,896, 4,294,828, 4,277,476, 4,267,328, 4,264,499, 4,231,930, 4,194,009,
4,188,388, 4,148,796, 4,128,717, 4,062,858, 4,031,226, 4,020,072, 4,018,895, 4,018,779,
4,013,672, 3,994,898, 3,968,125, 3,939,152, 3,928,356, 3,880,834, 3,668,210;

- 5 [151] cardiovascular agents, as disclosed in U.S. Pat. Nos. 4,966,967, 5,661,129, 5,552,411,
5,332,737, 5,389,675, 5,198,449, 5,079,247, 4,966,967, 4,874,760, 4,954,526, 5,051,423,
4,888,335, 4,853,391, 4,906,634, 4,775,757, 4,727,072, 4,542,160, 4,522,949, 4,524,151,
4,525,479, 4,474,804, 4,520,026, 4,520,026, 5,869,478, 5,859,239, 5,837,702, 5,807,889,
5,731,322, 5,726,171, 5,723,457, 5,705,523, 5,696,111, 5,691,332, 5,679,672, 5,661,129,
10 5,654,294, 5,646,276, 5,637,586, 5,631,251, 5,612,370, 5,612,323, 5,574,037, 5,563,170,
5,552,411, 5,552,397, 5,547,966, 5,482,925, 5,457,118, 5,414,017, 5,414,013, 5,401,758,
5,393,771, 5,362,902, 5,332,737, 5,310,731, 5,260,444, 5,223,516, 5,217,958, 5,208,245,
5,202,330, 5,198,449, 5,189,036, 5,185,362, 5,140,031, 5,128,349, 5,116,861, 5,079,247,
5,070,099, 5,061,813, 5,055,466, 5,051,423, 5,036,065, 5,026,712, 5,011,931, 5,006,542,
15 4,981,843, 4,977,144, 4,971,984, 4,966,967, 4,959,383, 4,954,526, 4,952,692, 4,939,137,
4,906,634, 4,889,866, 4,888,335, 4,883,872, 4,883,811, 4,847,379, 4,835,157, 4,824,831,
4,780,538, 4,775,757, 4,774,239, 4,771,047, 4,769,371, 4,767,756, 4,762,837, 4,753,946,
4,752,616, 4,749,715, 4,738,978, 4,735,962, 4,734,426, 4,734,425, 4,734,424, 4,730,052,
4,727,072, 4,721,796, 4,707,550, 4,704,382, 4,703,120, 4,681,970, 4,681,882, 4,670,560,
20 4,670,453, 4,668,787, 4,663,337, 4,663,336, 4,661,506, 4,656,267, 4,656,185, 4,654,357,
4,654,356, 4,654,355, 4,654,335, 4,652,578, 4,652,576, 4,650,874, 4,650,797, 4,649,139,
4,647,585, 4,647,573, 4,647,565, 4,647,561, 4,645,836, 4,639,461, 4,638,012, 4,638,011,
4,632,931, 4,631,283, 4,628,095, 4,626,548, 4,614,825, 4,611,007, 4,611,006, 4,611,005,
4,609,671, 4,608,386, 4,607,049, 4,607,048, 4,595,692, 4,593,042, 4,593,029, 4,591,603,
25 4,588,743, 4,588,742, 4,588,741, 4,582,854, 4,575,512, 4,568,762, 4,560,698, 4,556,739,
4,556,675, 4,555,571, 4,555,570, 4,555,523, 4,550,120, 4,542,160, 4,542,157, 4,542,156,
4,542,155, 4,542,151, 4,537,981, 4,537,904, 4,536,514, 4,536,513, 4,533,673, 4,526,901,
4,526,900, 4,525,479, 4,524,151, 4,522,949, 4,521,539, 4,520,026, 4,517,188, 4,482,562,
4,474,804, 4,474,803, 4,472,411, 4,466,979, 4,463,015, 4,456,617, 4,456,616, 4,456,615,
30 4,418,076, 4,416,896, 4,252,815, 4,220,594, 4,190,587, 4,177,280, 4,164,586, 4,151,297,
4,145,443, 4,143,054, 4,123,550, 4,083,968, 4,076,834, 4,064,259, 4,064,258, 4,064,257,
4,058,620, 4,001,421, 3,993,639, 3,991,057, 3,982,010, 3,980,652, 3,968,117, 3,959,296,

3,951,950, 3,933,834, 3,925,369, 3,923,818, 3,898,210, 3,897,442, 3,897,441, 3,886,157, 3,883,540, 3,873,715, 3,867,383, 3,873,715, 3,867,383, 3,691,216, 3,624,126;

[152] antimicrobial agents as disclosed in U.S. Pat. Nos. 5,902,594, 5,874,476, 5,874,436, 5,859,027, 5,856,320, 5,854,242, 5,811,091, 5,786,350, 5,783,177, 5,773,469, 5,762,919, 5,753,715, 5,741,526, 5,709,870, 5,707,990, 5,696,117, 5,684,042, 5,683,709, 5,656,591, 5,643,971, 5,643,950, 5,610,196, 5,608,056, 5,604,262, 5,595,742, 5,576,341, 5,554,373, 5,541,233, 5,534,546, 5,534,508, 5,514,715, 5,508,417, 5,464,832, 5,428,073, 5,428,016, 5,424,396, 5,399,553, 5,391,544, 5,385,902, 5,359,066, 5,356,803, 5,354,862, 5,346,913, 5,302,592, 5,288,693, 5,266,567, 5,254,685, 5,252,745, 5,209,930, 5,196,441, 5,190,961, 5,175,160, 5,157,051, 5,096,700, 5,093,342, 5,089,251, 5,073,570, 5,061,702, 5,037,809, 5,036,077, 5,010,109, 4,970,226, 4,916,156, 4,888,434, 4,870,093, 4,855,318, 4,784,991, 4,746,504, 4,686,221, 4,599,228, 4,552,882, 4,492,700, 4,489,098, 4,489,085, 4,487,776, 4,479,953, 4,477,448, 4,474,807, 4,470,994, 4,370,484, 4,337,199, 4,311,709, 4,308,283, 4,304,910, 4,260,634, 4,233,311, 4,215,131, 4,166,122, 4,141,981, 4,130,664, 4,089,977, 4,089,900, 4,069,341, 4,055,655, 4,049,665, 4,044,139, 4,002,775, 3,991,201, 3,966,968, 3,954,868, 3,936,393, 3,917,476, 3,915,889, 3,867,548, 3,865,748, 3,867,548, 3,865,748, 3,783,160, 3,764,676, 3,764,677;

[153] anti-inflammatory agents as disclosed in U.S. Pat. Nos. 5,872,109, 5,837,735, 5,827,837, 5,821,250, 5,814,648, 5,780,026, 5,776,946, 5,760,002, 5,750,543, 5,741,798, 5,739,279, 5,733,939, 5,723,481, 5,716,967, 5,688,949, 5,686,488, 5,686,471, 5,686,434, 5,684,204, 5,684,041, 5,684,031, 5,684,002, 5,677,318, 5,674,891, 5,672,620, 5,665,752, 5,656,661, 5,635,516, 5,631,283, 5,622,948, 5,618,835, 5,607,959, 5,593,980, 5,593,960, 5,580,888, 5,552,424, 5,552,422, 5,516,764, 5,510,361, 5,508,026, 5,500,417, 5,498,405, 5,494,927, 5,476,876, 5,472,973, 5,470,885, 5,470,842, 5,464,856, 5,464,849, 5,462,952, 5,459,151, 5,451,686, 5,444,043, 5,436,265, 5,432,181, RE034918, 5,393,756, 5,380,738, 5,376,670, 5,360,811, 5,354,768, 5,348,957, 5,347,029, 5,340,815, 5,338,753, 5,324,648, 5,319,099, 5,318,971, 5,312,821, 5,302,597, 5,298,633, 5,298,522, 5,298,498, 5,290,800, 5,290,788, 5,284,949, 5,280,045, 5,270,319, 5,266,562, 5,256,680, 5,250,700, 5,250,552, 5,248,682, 5,244,917, 5,240,929, 5,234,939, 5,234,937, 5,232,939, 5,225,571, 5,225,418, 5,220,025, 5,212,189, 5,212,172, 5,208,250, 5,204,365, 5,202,350, 5,196,431, 5,191,084, 5,187,175, 5,185,326, 5,183,906, 5,177,079, 5,171,864, 5,169,963, 5,155,122, 5,143,929, 5,143,928, 5,143,927, 5,124,455, 5,124,347, 5,114,958, 5,112,846, 5,104,656, 5,098,613, 5,095,037, 5,095,019, 5,086,064, 5,081,261, 5,081,147, 5,081,126, 5,075,330, 5,066,668,

5,059,602, 5,043,457, 5,037,835, 5,037,811, 5,036,088, 5,013,850, 5,013,751, 5,013,736,
5,006,542, 4,992,448, 4,992,447, 4,988,733, 4,988,728, 4,981,865, 4,962,119, 4,959,378,
4,954,519, 4,945,099, 4,942,236, 4,931,457, 4,927,835, 4,912,248, 4,910,192, 4,904,786,
4,904,685, 4,904,674, 4,904,671, 4,897,397, 4,895,953, 4,891,370, 4,870,210, 4,859,686,
5 4,857,644, 4,853,392, 4,851,412, 4,847,303, 4,847,290, 4,845,242, 4,835,166, 4,826,990,
4,803,216, 4,801,598, 4,791,129, 4,788,205, 4,778,818, 4,775,679, 4,772,703, 4,767,776,
4,764,525, 4,760,051, 4,748,153, 4,725,616, 4,721,712, 4,713,393, 4,708,966, 4,695,571,
4,686,235, 4,686,224, 4,680,298, 4,678,802, 4,652,564, 4,644,005, 4,632,923, 4,629,793,
4,614,741, 4,599,360, 4,596,828, 4,595,694, 4,595,686, 4,594,357, 4,585,755, 4,579,866,
10 4,578,390, 4,569,942, 4,567,201, 4,563,476, 4,559,348, 4,558,067, 4,556,672, 4,556,669,
4,539,326, 4,537,903, 4,536,503, 4,518,608, 4,514,415, 4,512,990, 4,501,755, 4,495,197,
4,493,839, 4,465,687, 4,440,779, 4,440,763, 4,435,420, 4,412,995, 4,400,534, 4,355,034,
4,335,141, 4,322,420, 4,275,064, 4,244,963, 4,235,908, 4,234,593, 4,226,887, 4,201,778,
4,181,720, 4,173,650, 4,173,634, 4,145,444, 4,128,664, 4,125,612, 4,124,726, 4,124,707,
15 4,117,135, 4,027,031, 4,024,284, 4,021,553, 4,021,550, 4,018,923, 4,012,527, 4,011,326,
3,998,970, 3,998,954, 3,993,763, 3,991,212, 3,984,405, 3,978,227, 3,978,219, 3,978,202,
3,975,543, 3,968,224, 3,959,368, 3,949,082, 3,949,081, 3,947,475, 3,936,450, 3,934,018,
3,930,005, 3,857,955, 3,856,962, 3,821,377, 3,821,401, 3,789,121, 3,789,123, 3,726,978,
3,694,471, 3,691,214, 3,678,169, 3,624,216;
20 [154] immunosuppressive agents, as disclosed in U.S. Pat. Nos. 4,450,159, 4,450,159,
5,905,085, 5,883,119, 5,880,280, 5,877,184, 5,874,594, 5,843,452, 5,817,672, 5,817,661,
5,817,660, 5,801,193, 5,776,974, 5,763,478, 5,739,169, 5,723,466, 5,719,176, 5,696,156,
5,695,753, 5,693,648, 5,693,645, 5,691,346, 5,686,469, 5,686,424, 5,679,705, 5,679,640,
5,670,504, 5,665,774, 5,665,772, 5,648,376, 5,639,455, 5,633,277, 5,624,930, 5,622,970,
25 5,605,903, 5,604,229, 5,574,041, 5,565,560, 5,550,233, 5,545,734, 5,540,931, 5,532,248,
5,527,820, 5,516,797, 5,514,688, 5,512,687, 5,506,233, 5,506,228, 5,494,895, 5,484,788,
5,470,857, 5,464,615, 5,432,183, 5,431,896, 5,385,918, 5,349,061, 5,344,925, 5,330,993,
5,308,837, 5,290,783, 5,290,772, 5,284,877, 5,284,840, 5,273,979, 5,262,533, 5,260,300,
5,252,732, 5,250,678, 5,247,076, 5,244,896, 5,238,689, 5,219,884, 5,208,241, 5,208,228,
30 5,202,332, 5,192,773, 5,189,042, 5,169,851, 5,162,334, 5,151,413, 5,149,701, 5,147,877,
5,143,918, 5,138,051, 5,093,338, 5,091,389, 5,068,323, 5,068,247, 5,064,835, 5,061,728,
5,055,290, 4,981,792, 4,810,692, 4,410,696, 4,346,096, 4,342,769, 4,317,825, 4,256,766,
4,180,588, 4,000,275, 3,759,921;

- [155] analgesic agents, as disclosed in U.S. Pat. Nos. 5,292,736, 5,688,825, 5,554,789, 5,455,230, 5,292,736, 5,298,522, 5,216,165, 5,438,064, 5,204,365, 5,017,578, 4,906,655, 4,906,655, 4,994,450, 4,749,792, 4,980,365, 4,794,110, 4,670,541, 4,737,493, 4,622,326, 4,536,512, 4,719,231, 4,533,671, 4,552,866, 4,539,312, 4,569,942, 4,681,879, 4,511,724, 5 4,556,672, 4,721,712, 4,474,806, 4,595,686, 4,440,779, 4,434,175, 4,608,374, 4,395,402, 4,400,534, 4,374,139, 4,361,583, 4,252,816, 4,251,530, 5,874,459, 5,688,825, 5,554,789, 5,455,230, 5,438,064, 5,298,522, 5,216,165, 5,204,365, 5,030,639, 5,017,578, 5,008,264, 4,994,450, 4,980,365, 4,906,655, 4,847,290, 4,844,907, 4,794,110, 4,791,129, 4,774,256, 4,749,792, 4,737,493, 4,721,712, 4,719,231, 4,681,879, 4,670,541, 4,667,039, 4,658,037, 10 4,634,708, 4,623,648, 4,622,326, 4,608,374, 4,595,686, 4,594,188, 4,569,942, 4,556,672, 4,552,866, 4,539,312, 4,536,512, 4,533,671, 4,511,724, 4,440,779, 4,434,175, 4,400,534, 4,395,402, 4,391,827, 4,374,139, 4,361,583, 4,322,420, 4,306,097, 4,252,816, 4,251,530, 4,244,955, 4,232,018, 4,209,520, 4,164,514, 4,147,872, 4,133,819, 4,124,713, 4,117,012, 4,064,272, 4,022,836, 3,966,944;
- 15 [156] cholinergic agents, as disclosed in U.S. Pat. Nos. 5,219,872, 5,219,873, 5,073,560, 5,073,560, 5,346,911, 5,424,301, 5,073,560, 5,219,872, 4,900,748, 4,786,648, 4,798,841, 4,782,071, 4,710,508, 5,482,938, 5,464,842, 5,378,723, 5,346,911, 5,318,978, 5,219,873, 5,219,872, 5,084,281, 5,073,560, 5,002,955, 4,988,710, 4,900,748, 4,798,841, 4,786,648, 4,782,071, 4,745,123, 4,710,508;
- 20 [157] adrenergic agents, as disclosed in U.S. Pat. Nos. 5,091,528, 5,091,528, 4,835,157, 5,708,015, 5,594,027, 5,580,892, 5,576,332, 5,510,376, 5,482,961, 5,334,601, 5,202,347, 5,135,926, 5,116,867, 5,091,528, 5,017,618, 4,835,157, 4,829,086, 4,579,867, 4,568,679, 4,469,690, 4,395,559, 4,381,309, 4,363,808, 4,343,800, 4,329,289, 4,314,943, 4,311,708, 4,304,721, 4,296,117, 4,285,873, 4,281,189, 4,278,608, 4,247,710, 4,145,550, 4,145,425, 25 4,139,535, 4,082,843, 4,011,321, 4,001,421, 3,982,010, 3,940,407, 3,852,468, 3,832,470;
- [158] antihistamine agents, as disclosed in U.S. Pat. Nos. 5,874,479, 5,863,938, 5,856,364, 5,770,612, 5,702,688, 5,674,912, 5,663,208, 5,658,957, 5,652,274, 5,648,380, 5,646,190, 5,641,814, 5,633,285, 5,614,561, 5,602,183, 4,923,892, 4,782,058, 4,393,210, 4,180,583, 3,965,257, 3,946,022, 3,931,197;
- 30 [159] steroidal agents, as disclosed in U.S. Pat. Nos. 5,863,538, 5,855,907, 5,855,866, 5,780,592, 5,776,427, 5,651,987, 5,346,887, 5,256,408, 5,252,319, 5,209,926, 4,996,335, 4,927,807, 4,910,192, 4,710,495, 4,049,805, 4,004,005, 3,670,079, 3,608,076, 5,892,028,

5,888,995, 5,883,087, 5,880,115, 5,869,475, 5,866,558, 5,861,390, 5,861,388, 5,854,235,
 5,837,698, 5,834,452, 5,830,886, 5,792,758, 5,792,757, 5,763,361, 5,744,462, 5,741,787,
 5,741,786, 5,733,899, 5,731,345, 5,723,638, 5,721,226, 5,712,264, 5,712,263, 5,710,144,
 5,707,984, 5,705,494, 5,700,793, 5,698,720, 5,698,545, 5,696,106, 5,677,293, 5,674,861,
 5 5,661,141, 5,656,621, 5,646,136, 5,637,691, 5,616,574, 5,614,514, 5,604,215, 5,604,213,
 5,599,807, 5,585,482, 5,565,588, 5,563,259, 5,563,131, 5,561,124, 5,556,845, 5,547,949,
 5,536,714, 5,527,806, 5,506,354, 5,506,221, 5,494,907, 5,491,136, 5,478,956, 5,426,179,
 5,422,262, 5,391,776, 5,382,661, 5,380,841, 5,380,840, 5,380,839, 5,373,095, 5,371,078,
 5,352,809, 5,344,827, 5,344,826, 5,338,837, 5,336,686, 5,292,906, 5,292,878, 5,281,587,
 10 5,272,140, 5,244,886, 5,236,912, 5,232,915, 5,219,879, 5,218,109, 5,215,972, 5,212,166,
 5,206,415, 5,194,602, 5,166,201, 5,166,055, 5,126,488, 5,116,829, 5,108,996, 5,099,037,
 5,096,892, 5,093,502, 5,086,047, 5,084,450, 5,082,835, 5,081,114, 5,053,404, 5,041,433,
 5,041,432, 5,034,548, 5,032,586, 5,026,882, 4,996,335, 4,975,537, 4,970,205, 4,954,446,
 4,950,428, 4,946,834, 4,937,237, 4,921,846, 4,920,099, 4,910,226, 4,900,725, 4,892,867,
 15 4,888,336, 4,885,280, 4,882,322, 4,882,319, 4,882,315, 4,874,855, 4,868,167, 4,865,767,
 4,861,875, 4,861,765, 4,861,763, 4,847,014, 4,774,236, 4,753,932, 4,711,856, 4,710,495,
 4,701,450, 4,701,449, 4,689,410, 4,680,290, 4,670,551, 4,664,850, 4,659,516, 4,647,410,
 4,634,695, 4,634,693, 4,588,530, 4,567,000, 4,560,557, 4,558,041, 4,552,871, 4,552,868,
 4,541,956, 4,519,946, 4,515,787, 4,512,986, 4,502,989, 4,495,102;

20 [160] the disclosures of all the above of which are herein incorporated by reference.

[161] The drug moiety of the conjugate may be the whole compound or a binding fragment
 or portion thereof that retains its affinity and specificity for the target of interest while having
 a linkage site for covalent bonding to the presenter protein ligand or linker. The conjugates
 of such drugs may be used for the same disorders, diseases, and indications as the drugs
 25 themselves.

M. P97 and modulators

[162] In one embodiment, the active agent is conjugated to p97 or a a modulator or ligand
 of the LRP receptor family (e.g. LRP1, LRP1B), or is an antibody which is capable of
 specifically binding to p97 or the modulator, such as an antibody to p97. In a further
 30 embodiment, the agent may be a substance having therapeutic activity such as a growth factor
 or lymphokine, enzyme or drug. The invention also relates to a method of delivering an
 active agent across the blood brain barrier comprising administering such a conjugate.

[163] In one embodiment, the p97 protein is soluble. p97 proteins as taught in U.S. Patent No. 5,981,194 are particularly preferred. The p97 may be a human p97 protein or fragment thereof; the p97 may be from a mammal such as a mouse. Murine p97 is disclosed in WO 01/59549 A2 which is herein incorporated by reference in its entirety.

5 [164] "p97" as used in the compositions of the invention, includes membrane bound p97 (*i.e.*, p97 linked to GPI or other lipids), soluble p97, cleaved p97, analogs of p97 which are equivalents of p97 (having greater than 40%, 60%, 80%, or 90% homology at the peptide sequence level, including allelic variants of p97), human, mouse, chicken and/or rabbit p97, and derivatives, portions, or fragments thereof. p97 may be in the form of acidic or basic
10 salts, or in neutral form. In addition, individual amino acid residues may be modified, such as by oxidation or reduction. Various substitutions, deletions, or additions may be made to the amino acid or DNA nucleic acid sequences, the net effect of which is to retain or improve upon the desired biological activity of p97. Due to code degeneracy, for example, there may be considerable variation in nucleotide sequences encoding the same amino acid sequence.
15 As used herein, p97 also includes fragments of p97, including any portion of p97 or its biologically equivalent analogs that contain a sufficient portion of p97 and homology to the corresponding native p97 amino acid sequence to enable it to retain or improve upon the desired biological activities of p97. In other aspects, the invention is drawn to p97 conjugates which have only minor substitutions in the amino acid sequence which do not substantially
20 affect its receptor binding or transcytosis properties.

[165] Preferred chemotherapeutic agents for use in p97-chemotherapeutic agent conjugates of the invention include all drugs which may be useful for treating brain tumours or other neoplasia in or around the brain, either in the free form, or, if not so useful in the free form, then useful when linked to p97. Such chemotherapeutic agents include adriamycin (also
25 known as doxorubicin), cisplatin, paclitaxel, analogs thereof, and other chemotherapeutic agents which demonstrate activity against tumours *ex vivo* and *in vivo*. Such chemotherapeutic agents also include alkylating agents, antimetabolites, natural products (such as vinca alkaloids, epidophyllotoxins, antibiotics, enzymes and biological response modifiers), topoisomerase inhibitors, microtubule inhibitors, spindle poisons, hormones and
30 antagonists, and miscellaneous agents such as platinum coordination complexes, anthracendiones, substituted ureas, *etc.* those of skill in the art will know of other chemotherapeutic agents.

[166] p97-chemotherapeutic agents can comprise one or more compound moieties linked to p97. For example, conjugation reactions may conjugate from 1 to 10 or more molecules of adriamycin to a single p97 molecule. Several atoms of gold or iodine can be conjugated to a single p97 polypeptide. These formulations can be employed as mixtures, or they may be purified into specific p97:compound (mol:mol) formulations. Those skilled in the art are able to determine which format and which mol:mol ratio is preferred. Further, mixtures of compounds may be linked to p97, such as the p97-adriamycin-cisplatinum composition set out in the examples. These p97-chemotherapeutic agents may consist of a range of mol:mol ratios. These, too, may be separated into purified mixtures or they may be employed in aggregate.

[167] The compositions of the invention may also be used for delivering an agent across the blood eye barrier or blood placenta barrier

[168] The compositions of the invention may also comprise a transcytosing or endocytosing ligand of the LRP1 or LRP1B receptor conjugated to an active agent.

N. Labels

[169] In some embodiments, the conjugate or modulator or LRP ligand according to the invention is labeled to facilitate its detection. A "label" or a "detectable moiety" is a composition detectable by spectroscopic, photochemical, biochemical, immunochemical, chemical, or other physical means. For example, labels suitable for use in the present invention include, for example, radioactive labels (*e.g.*, ^{32}P), fluorophores (*e.g.*, fluorescein), electron-dense reagents, enzymes (*e.g.*, as commonly used in an ELISA), biotin, digoxigenin, or haptens and proteins which can be made detectable, *e.g.*, by incorporating a radiolabel into the hapten or peptide, or used to detect antibodies specifically reactive with the hapten or peptide.

[170] As noted above, depending on the screening assay employed, the drug, the linker or the p97 or modulator or ligand portion of a conjugate may be labeled. The particular label or detectable group used is not a critical aspect of the invention, as long as it does not significantly interfere with the biological activity of the conjugate. The detectable group can be any material having a detectable physical or chemical property. Thus, a label is any composition detectable by spectroscopic, photochemical, biochemical, immunochemical, electrical, optical or chemical means.

[171] Examples of labels suitable for use in the present invention include, but are not limited to, fluorescent dyes (*e.g.*, fluorescein isothiocyanate, Texas red, rhodamine, and the like), radiolabels (*e.g.*, ^3H , ^{125}I , ^{35}S , ^{14}C , or ^{32}P), enzymes (*e.g.*, horse radish peroxidase, alkaline phosphatase and others commonly used in an ELISA), and colorimetric labels such as colloidal gold or colored glass or plastic beads (*e.g.*, polystyrene, polypropylene, latex, *etc.*).

[172] The label may be coupled directly or indirectly to the desired component of the assay according to methods well known in the art. Preferably, the label in one embodiment is covalently bound to the biopolymer using an isocyanate reagent for conjugating an active agent according to the invention. In one aspect of the invention, the bifunctional isocyanate reagents of the invention can be used to conjugate a label to a biopolymer to form a label biopolymer conjugate without an active agent attached thereto. The label biopolymer conjugate may be used as an intermediate for the synthesis of a labeled conjugate according to the invention or may be used to detect the biopolymer conjugate. As indicated above, a wide variety of labels can be used, with the choice of label depending on sensitivity required, ease of conjugation with the desired component of the assay, stability requirements, available instrumentation, and disposal provisions. Non-radioactive labels are often attached by indirect means. Generally, a ligand molecule (*e.g.*, biotin) is covalently bound to the molecule. The ligand then binds to another molecules (*e.g.*, streptavidin) molecule, which is either inherently detectable or covalently bound to a signal system, such as a detectable enzyme, a fluorescent compound, or a chemiluminescent compound.

[173] The conjugates can also be conjugated directly to signal generating compounds, *e.g.*, by conjugation with an enzyme or fluorophore. Enzymes suitable for use as labels include, but are not limited to, hydrolases, particularly phosphatases, esterases and glycosidases, or oxidotases, particularly peroxidases. Fluorescent compounds, *i.e.*, fluorophores, suitable for use as labels include, but are not limited to, fluorescein and its derivatives, rhodamine and its derivatives, dansyl, umbelliferone, *etc.* Further examples of suitable fluorophores include, but are not limited to, eosin, TRITC-amine, quinine, fluorescein W, acridine yellow, lissamine rhodamine, B sulfonyl chloride erythroscein, ruthenium (tris, bipyridinium), Texas Red, nicotinamide adenine dinucleotide, flavin adenine dinucleotide, *etc.* Chemiluminescent compounds suitable for use as labels include, but are not limited to, luciferin and 2,3-dihydrophthalazinediones, *e.g.*, luminol. For a review of various labeling or signal producing

systems that can be used in the methods of the present invention, *see* U.S. Patent No. 4,391,904.

[174] Means of detecting labels are well known to those of skill in the art. Thus, for example, where the label is a radioactive label, means for detection include a scintillation counter or photographic film as in autoradiography. Where the label is a fluorescent label, it may be detected by exciting the fluorochrome with the appropriate wavelength of light and detecting the resulting fluorescence. The fluorescence may be detected visually, by the use of electronic detectors such as charge coupled devices (CCDs) or photomultipliers and the like. Similarly, enzymatic labels may be detected by providing the appropriate substrates for the enzyme and detecting the resulting reaction product. Colorimetric or chemiluminescent labels may be detected simply by observing the color associated with the label. Other labeling and detection systems suitable for use in the methods of the present invention will be readily apparent to those of skill in the art. Such labeled modulators and ligands may be used in the diagnosis of a disease or health condition.

O. Methods of Using, Pharmaceutical Compositions, and their Administration

[175] The term "pharmaceutically acceptable carrier" encompasses any of the standard pharmaceutical carriers, buffers and excipients, including phosphate-buffered saline solution, water, and emulsions (such as an oil/water or water/oil emulsion), and various types of wetting agents and/or adjuvants. Suitable pharmaceutical carriers and their formulations are described in Remington's Pharmaceutical Sciences (Mack Publishing Co., Easton, 19th ed. 1995). Preferred pharmaceutical carriers depend upon the intended mode of administration of the active agent. Typical modes of administration are described below.

[176] The term "effective amount" means a dosage sufficient to produce a desired result on a health condition, pathology, disease of a subject or for a diagnostic purpose. The desired result may comprise a subjective or objective improvement in the recipient of the dosage.

[177] A "prophylactic treatment" is a treatment administered to a subject who does not exhibit signs of a disease or exhibits only early signs of a disease, wherein treatment is administered for the purpose of decreasing the risk of developing a pathology. The conjugate compounds of the invention may be given as a prophylactic treatment.

[178] A "therapeutic treatment" is a treatment administered to a subject who exhibits signs of pathology, wherein treatment is administered for the purpose of diminishing or eliminating

those pathological signs. The conjugate compounds, modulators, and ligands of the invention may be given as a prophylactic treatment or for diagnosis.

[179] The term "composition", as in pharmaceutical composition, is intended to encompass a product comprising the active ingredient(s), and the inert ingredient(s) that make up the carrier, as well as any product which results, directly or indirectly, from combination, complexation or aggregation of any two or more of the ingredients, or from dissociation of one or more of the ingredients, or from other types of reactions or interactions of one or more of the ingredients. Accordingly, the pharmaceutical compositions of the present invention encompass any composition made by admixing a conjugate compound of the present invention and a pharmaceutically acceptable carrier. The term "pharmaceutical composition" indicates a composition suitable for pharmaceutical use in a subject, including an animal or human. A pharmaceutical composition generally comprises an effective amount of a conjugate, modulator, or LRP ligand or LRP1B ligand and a pharmaceutically acceptable carrier.

[180] The conjugates, modulators, and LRP or LRP1B ligands may be administered by a variety of routes. For oral preparations, the conjugates can be used alone or in combination with appropriate additives to make tablets, powders, granules or capsules, for example, with conventional additives, such as lactose, mannitol, corn starch or potato starch; with binders, such as crystalline cellulose, cellulose derivatives, acacia, corn starch or gelatins; with disintegrators, such as corn starch, potato starch or sodium carboxymethylcellulose; with lubricants, such as talc or magnesium stearate; and if desired, with diluents, buffering agents, moistening agents, preservatives and flavoring agents.

[181] The conjugates, modulators, and LRP or LRP1B ligands can be formulated into preparations for injection by dissolving, suspending or emulsifying them in an aqueous or nonaqueous solvent, such as vegetable or other similar oils, synthetic aliphatic acid glycerides, esters of higher aliphatic acids or propylene glycol; and if desired, with conventional additives such as solubilizers, isotonic agents, suspending agents, emulsifying agents, stabilizers and preservatives.

[182] The conjugates, modulators, and LRP or LRP1B ligands can be utilized in aerosol formulation to be administered via inhalation. The compounds of the present invention can be formulated into pressurized acceptable propellants such as dichlorodifluoromethane, propane, nitrogen and the like.

[183] Furthermore, the conjugates, modulators, and LRP or LRP1B ligands can be made into suppositories by mixing with a variety of bases such as emulsifying bases or water-soluble bases. The compounds of the present invention can be administered rectally via a suppository. The suppository can include vehicles such as cocoa butter, carbowaxes and polyethylene glycols, which melt at body temperature, yet are solidified at room temperature.

[184] Unit dosage forms of the conjugate, modulator, and LRP or LRP1B ligand for oral or rectal administration such as syrups, elixirs, and suspensions may be provided wherein each dosage unit, for example, teaspoonful, tablespoonful, tablet or suppository, contains a predetermined amount of the composition containing active agent. Similarly, unit dosage forms for injection or intravenous administration may comprise the conjugate in a composition as a solution in sterile water, normal saline or another pharmaceutically acceptable carrier. The term "unit dosage form," as used herein, refers to physically discrete units suitable as unitary dosages for human and animal subjects, each unit containing a predetermined quantity of compounds of the present invention calculated in an amount sufficient to produce the desired effect in association with a pharmaceutically acceptable diluent, carrier or vehicle. The specifications for the novel unit dosage forms of the present invention depend on the particular conjugate employed and the effect to be achieved, and the pharmacodynamics associated with each compound in the host.

[185] In practical use, the conjugate, modulator, and LRP or LRP1B ligand according to the invention can be combined as the active ingredient in intimate admixture with a pharmaceutical carrier according to conventional pharmaceutical compounding techniques. The carrier may take a wide variety of forms depending on the form of preparation desired for administration, *e.g.*, oral or parenteral (including intravenous). In preparing the compositions for oral dosage form, any of the usual pharmaceutical media may be employed, such as, for example, water, glycols, oils, alcohols, flavoring agents, preservatives, coloring agents and the like in the case of oral liquid preparations, such as, for example, suspensions, elixirs and solutions; or carriers such as starches, sugars, microcrystalline cellulose, diluents, granulating agents, lubricants, binders, disintegrating agents and the like in the case of oral solid preparations such as, for example, powders, hard and soft capsules and tablets, with the solid oral preparations being preferred over the liquid preparations.

[186] With respect to transdermal routes of administration, methods for transdermal administration of drugs are disclosed in Remington's Pharmaceutical Sciences, 17th Edition, (Gennaro et al. Eds., Mack Publishing Co., 1985). Dermal or skin patches are a preferred

means for transdermal delivery of the conjugates, modulators, and LRP or LRP1B ligands of the invention. Patches preferably provide an absorption enhancer such as DMSO to increase the absorption of the compounds. Other methods for transdermal drug delivery are disclosed in U.S. Patents No. 5,962,012, 6,261,595, and 6,261,595. Each of which is incorporated by
5 reference in its entirety.

[187] The pharmaceutically acceptable excipients, such as vehicles, adjuvants, carriers or diluents, are commercially available. Moreover, pharmaceutically acceptable auxiliary substances, such as pH adjusting and buffering agents, tonicity adjusting agents, stabilizers, wetting agents and the like, are commercially available.

10 [188] Those of skill will readily appreciate that dose levels can vary as a function of the specific compound, the severity of the symptoms and the susceptibility of the subject to side effects. Preferred dosages for a given compound are readily determinable by those of skill in the art by a variety of means.

[189] In each of these aspects, the compositions include, but are not limited to,
15 compositions suitable for oral, rectal, topical, parenteral (including subcutaneous, intramuscular, and intravenous), pulmonary (nasal or buccal inhalation), or nasal administration, although the most suitable route in any given case will depend in part on the nature and severity of the conditions being treated and on the nature of the active ingredient. Exemplary routes of administration are the oral and intravenous routes. The compositions
20 may be conveniently presented in unit dosage form and prepared by any of the methods well-known in the art of pharmacy.

[190] In practical use, the compounds (e.g., LRP or LRP1B ligand conjugates, LRP or LRP1B modulator conjugates, LRP or LRP1B ligand-fusion proteins, LRP or LRP1B modulators, and LRP or LRP1B ligands) according to the invention can be combined as the
25 active ingredient in intimate admixture with a pharmaceutical carrier according to conventional pharmaceutical compounding techniques. The carrier may take a wide variety of forms depending on the form of preparation desired for administration, e.g., oral or parenteral (including intravenous). In preparing the compositions for oral dosage form, any of the usual pharmaceutical media may be employed, such as, for example, water, glycols,
30 oils, alcohols, flavoring agents, preservatives, coloring agents and the like in the case of oral liquid preparations, such as, for example, suspensions, elixirs and solutions; or carriers such as starches, sugars, microcrystalline cellulose, diluents, granulating agents, lubricants,

binders, disintegrating agents and the like in the case of oral solid preparations such as, for example, powders, hard and soft capsules and tablets, with the solid oral preparations being preferred over the liquid preparations.

[191] Because of their ease of administration, tablets and capsules represent the most advantageous oral dosage unit form in which case solid pharmaceutical carriers are obviously employed. If desired, tablets may be coated by standard aqueous or nonaqueous techniques. The percentage of an active compound in these compositions may, of course, be varied and may conveniently be between about 2 percent to about 60 percent of the weight of the unit.

[192] The conjugates, modulators, and ligands of the invention are useful for therapeutic, prophylactic and diagnostic intervention in animals, and in particular in humans. As described herein, the conjugates show preferential accumulation and/or release of the active agent in any target organ, compartment, or site depending upon the biopolymer used.

[193] Compositions of the present invention may be administered encapsulated in or attached to viral envelopes or vesicles, or incorporated into cells. Vesicles are micellular particles which are usually spherical and which are frequently lipidic. Liposomes are vesicles formed from a bilayer membrane. Suitable vesicles include, but are not limited to, unilamellar vesicles and multilamellar lipid vesicles or liposomes. Such vesicles and liposomes may be made from a wide range of lipid or phospholipid compounds, such as phosphatidylcholine, phosphatidic acid, phosphatidylserine, phosphatidylethanolamine, sphingomyelin, glycolipids, gangliosides, *etc.* using standard techniques, such as those described in, *e.g.*, U.S. Patent No. 4,394,448. Such vesicles or liposomes may be used to administer compounds intracellularly and to deliver compounds to the target organs. Controlled release of a p97-composition of interest may also be achieved using encapsulation (*see, e.g.*, U.S. Patent No. 5,186,941).

[194] Any route of administration which dilutes the conjugates, modulators, and LRP or LRP1B ligands composition into the blood stream, or at least outside of the blood-brain barrier, may be used. Preferably, the composition is administered peripherally, most preferably intravenously or by cardiac catheter. Intra-jugular and intra-carotid injections are also useful. Compositions may be administered locally or regionally, such as intra-peritoneally. In one aspect, compositions are administered with a suitable pharmaceutical diluent or carrier.

[195] Dosages to be administered will depend on individual needs, on the desired effect, the active agent used, the biopolymer and on the chosen route of administration. Preferred dosages of a conjugate range from about 0.2 pmol/kg to about 25 nmol/kg, and particularly preferred dosages range from 2-250 pmol/kg; alternatively, preferred doses of the conjugate may be in the range of 0.02 to 2000 mg/kg. These dosages will be influenced by the number of active agent or drug moieties associated with the biopolymer. Alternatively, dosages may be calculated based on the active agent administered.

[196] In preferred embodiment the conjugate comprises p97. For instance, doses of p97-adriamycin comprising from 0.005 to 100 mg/kg of adriamycin are also useful *in vivo*.

Particularly preferred is a dosage of p97-adriamycin comprising from 0.05 mg/kg to 20 mg/kg of adriamycin. Those skilled in the art can determine suitable doses for other compounds linked to p97 based on the recommended dosage used for the free form of the compound. p97 generally reduces the amount of drug needed to obtain the same effect.

[197] The p97-conjugates, modulators, and LRP1 or LRP1B ligands of the invention are useful for therapeutic, prophylactic and diagnostic intervention in animals, and in particular in humans. As described herein, p97-compounds show preferential accumulation in the lung, liver, kidney and spleen, and that they significantly reduce delivery of the compounds to the heart. Preferred medical indications for diagnostic uses include, for example, any condition associated with a target organ of interest (*e.g.*, lung, liver, kidney, spleen) or any condition that requires a cardiotoxic compound that would benefit by reducing its cardiotoxicity.

[198] The subject methods find use in the treatment of a variety of different disease conditions. In certain embodiments, of particular interest is the use of the subject methods in disease conditions where an active agent or drug having desired activity has been previously identified, but in which the active agent or drug is not targeted to the target site, area or compartment. With such active agents or drugs, the subject methods can be used to enhance the therapeutic efficacy and therapeutic index of active agent or drug.

[199] The specific disease conditions treatable by with the subject conjugates are as varied as the types of drug moieties that can be present in the conjugate. Thus, disease conditions include cellular proliferative diseases, such as neoplastic diseases, autoimmune diseases, cardiovascular diseases, hormonal abnormality diseases, degenerative diseases, diseases of aging, diseases of the central nervous system (*e.g.*, Alzheimer's disease, epilepsy,

hyperlipidemias), psychiatric diseases and conditions(e.g., schizophrenia, mood disorders such as depression and anxiety), infectious diseases, and the like.

[200] Treatment is meant to encompass any beneficial outcome to a subject associated with administration of a conjugate including a reduced likelihood of acquiring a disease, prevention of a disease, slowing, stopping or reversing, the progression of a disease or an amelioration of the symptoms associated with the disease condition afflicting the host, where amelioration or benefit is used in a broad sense to refer to at least a reduction in the magnitude of a parameter, e.g., symptom, associated with the pathological condition being treated, such as inflammation and pain associated therewith. As such, treatment also includes situations where the pathological condition, or at least symptoms associated therewith, are completely inhibited, e.g., prevented from happening, or stopped, e.g., terminated, such that the host no longer suffers from the pathological condition, or at least the symptoms that characterize the pathological condition.

[201] A variety of hosts or subjects are treatable according to the subject methods.

Generally such hosts are "mammals" or "mammalian," where these terms are used broadly to describe organisms which are within the class mammalia, including the orders carnivore (e.g., dogs and cats), rodentia (e.g., mice, guinea pigs, and rats), and primates (e.g., humans, chimpanzees, and monkeys). In many embodiments, the hosts will be humans.

[202] P. Combinatorial chemical libraries

[203] Recently, attention has focused on the use of combinatorial chemical libraries to assist in the generation of new chemical compound leads. A combinatorial chemical library is a collection of diverse chemical compounds generated by either chemical synthesis or biological synthesis by combining a number of chemical "building blocks" such as reagents. For example, a linear combinatorial chemical library such as a polypeptide library is formed by combining a set of chemical building blocks called amino acids in every possible way for a given compound length (i.e., the number of amino acids in a polypeptide compound). Millions of chemical compounds can be synthesized through such combinatorial mixing of chemical building blocks. For example, one commentator has observed that the systematic, combinatorial mixing of 100 interchangeable chemical building blocks results in the theoretical synthesis of 100 million tetrameric compounds or 10 billion pentameric compounds (Gallop et al. *J. Med. Chem.* 37(9):1233(1994)).

[204] Preparation and screening of combinatorial chemical libraries are well known to those of skill in the art. Such combinatorial chemical libraries include, but are not limited to, benzodiazepines (U.S. Pat. No. 5,288,514), diversomers such as hydantoins, benzodiazepines and dipeptides (Hobbs et al. *PNAS USA* 90: 6909(1993)), analogous organic syntheses of small compound libraries (Chen et al.) *J. Amer. Chem. Soc.* 116: 2661(1994), oligocarbamates (Cho, et al., *Science* 261: 1303(1993)), and/or peptidyl phosphonates (Campbell et al., *J. Org. Chem.* 59: 658(1994)), and small organic molecule libraries (see, e.g., benzodiazepines (Baum *C&EN*, Jan 18, page 33(1993)), thiazolidinones and metathiazanones (U.S. Patent 5,549,974), pyrrolidines (U.S. Patents 5,525,735 and 5,519,134), benzodiazepines (U.S. Patent 5,288,514), and the like.

[205] Devices for the preparation of combinatorial libraries are commercially available (see, e.g., 357 MPS, 390 MPS, Advanced Chem Tech, Louisville KY, Symphony, Rainin, Woburn, MA, 433A Applied Biosystems, Foster City, CA, 9050 Plus, Millipore, Bedford, MA).

[206] A number of well known robotic systems have also been developed for solution phase chemistries. These systems include automated workstations like the automated synthesis apparatus developed by Takeda Chemical Industries, LTD. (Osaka, Japan) and many robotic systems utilizing robotic arms (Zymate II, Zymark Corporation, Hopkinton, Mass.; Orca, HewlettPackard, Palo Alto, CA) which mimic the manual synthetic operations performed by a chemist. Any of the above devices are suitable for use with the present invention. The nature and implementation of modifications to these devices so that they can operate as discussed herein will be apparent to persons skilled in the relevant art. In addition, numerous combinatorial libraries are themselves commercially available (see, e.g., ComGenex, Princeton, N.J., Asinex, Moscow, Ru, Tripos, Inc., St. Louis, MO, ChemStar, Ltd., Moscow, RU, 3D Pharmaceuticals, Exton, PA, Martek Biosciences, Columbia, MD, etc.).

[207] In a preferred embodiment, the combinatorial chemistry provides variants of the known native or endogenous human ligands of the LRP and LRP1B receptors as candidate ligands, modulators, and conjugates for use according to the invention.

Q. High throughput assays of chemical libraries

[208] The assays for compounds described herein are amenable to high throughput screening. Preferred assays thus detect activation of transcription (i.e., activation of mRNA production) by the test compound(s), activation of protein expression by the test

compound(s), or binding to the gene product (e.g., expressed protein) by the test compound(s). The BiaCore method is one such means for rapidly screening compounds for binding activity.

[209] High throughput assays for the presence, absence, or quantification of particular protein products or binding assays are well known to those of skill in the art. Thus, for example, U.S. Patent 5,559,410 discloses high throughput screening methods for proteins, and U.S. Patents 5,576,220 and 5,541,061 disclose high throughput methods of screening for ligand/antibody binding.

[210] In addition, high throughput screening systems are commercially available (see, e.g., Zymark Corp., Hopkinton, MA; Air Technical Industries, Mentor, OH; Beckman Instruments, Inc. Fullerton, CA; Precision Systems, Inc., Natick, MA, etc.). These systems typically automate entire procedures including all sample and reagent pipetting, liquid dispensing, timed incubations, and final readings of the microplate in detector(s) appropriate for the assay. These configurable systems provide high throughput and rapid start up as well as a high degree of flexibility and customization. The manufacturers of such systems provide detailed protocols the various high throughput. Thus, for example, Zymark Corp. provides technical bulletins describing screening systems for detecting the modulation of gene transcription, ligand binding, and the like.

R. Conjugation and labeling

[211] One of ordinary skill in the art would know how to conjugate and active agent to a protein or peptide. Methods of conjugating active agents and labels to proteins are well known in the art. See, for instance, U.S. Patent No. 5,981,194. Many reagents and cross linkers can be used to prepare bioconjugates of an active agent and a biopolymer. See, for instance, Hermanson, GT et al. Bioconjugate Techniques, Academic Press, (1996).

Production of Chimeric Proteins

[212] The chimeric protein of the present invention can be produced using host cells expressing a single nucleic acid encoding the entire chimeric protein or more than one nucleic acid sequence, each encoding a domain of the chimeric protein and, optionally, an amino acid or amino acids which will serve to link the domains. The chimeric proteins can also be produced by chemical synthesis.

A. Host Cells

[213] Host cells used to produce chimeric proteins are bacterial, yeast, insect, non-mammalian vertebrate, or mammalian cells; the mammalian cells include, but are not limited to, hamster, monkey, chimpanzee, dog, cat, bovine, porcine, mouse, rat, rabbit, sheep and human cells. The host cells can be immortalized cells (a cell line) or non-immortalized (primary or secondary) cells and can be any of a wide variety of cell types, such as, but not limited to, fibroblasts, keratinocytes, epithelial cells (e.g., mammary epithelial cells, intestinal epithelial cells), ovary cells (e.g., Chinese hamster ovary or CHO cells), endothelial cells, glial cells, neural cells, formed elements of the blood (e.g., lymphocytes, bone marrow cells), muscle cells, hepatocytes and precursors of these somatic cell types.

[214] Cells which contain and express DNA or RNA encoding the chimeric protein are referred to herein as genetically modified cells. Mammalian cells which contain and express DNA or RNA encoding the chimeric protein are referred to as genetically modified mammalian cells. Introduction of the DNA or RNA into cells is by a known transfection method, such as electroporation, microinjection, microprojectile bombardment, calcium phosphate precipitation, modified calcium phosphate precipitation, cationic lipid treatment, photoporation, fusion methodologies, receptor mediated transfer, or polybrene precipitation. Alternatively, the DNA or RNA can be introduced by infection with a viral vector. Methods of producing cells, including mammalian cells, which express DNA or RNA encoding a chimeric protein are described in co-pending patent applications U.S. Ser. No. 08/334,797, entitled "In Vivo Protein Production and Delivery System for Gene Therapy", by Richard F Selden, Douglas A. Treco and Michael W. Heartlein (filed Nov. 4, 1994); U.S. Ser. No. 08/334,455, entitled "In Vivo Production and Delivery of Erythropoietin or Insulinotropin for Gene Therapy", by Richard F Selden, Douglas A. Treco and Michael W. Heartlein (filed Nov. 4, 1994) and U.S. Ser. No. 08/231,439, entitled "Targeted Introduction of DNA Into Primary or Secondary Cells and Their Use for Gene Therapy", by Douglas A. Treco, Michael W. Heartlein and Richard F Selden (filed Apr. 20, 1994). The teachings of each of these applications are expressly incorporated herein by reference.

B. Nucleic Acid Constructs

[215] A nucleic acid construct used to express the chimeric protein can be one which is expressed extrachromosomally (episomally) in the transfected mammalian cell or one which integrates, either randomly or at a pre-selected targeted site through homologous recombination, into the recipient cell's genome. A construct which is expressed extrachromosomally comprises, in addition to chimeric protein-encoding sequences,

sequences sufficient for expression of the protein in the cells and, optionally, for replication of the construct. It typically includes a promoter, chimeric protein-encoding DNA and a polyadenylation site. The DNA encoding the chimeric protein is positioned in the construct in such a manner that its expression is under the control of the promoter. Optionally, the
5 construct may contain additional components such as one or more of the following: a splice site, an enhancer sequence, a selectable marker gene under the control of an appropriate promoter, and an amplifiable marker gene under the control of an appropriate promoter.

[216] In those embodiments in which the DNA construct integrates into the cell's genome, it need include only the chimeric protein-encoding nucleic acid sequences. Optionally, it can
10 include a promoter and an enhancer sequence, a polyadenylation site or sites, a splice site or sites, nucleic acid sequences which encode a selectable marker or markers, nucleic acid sequences which encode an amplifiable marker and/or DNA homologous to genomic DNA in the recipient cell to target integration of the DNA to a selected site in the genome (targeting DNA or DNA sequences).

15 C. Cell Culture Methods

[217] Mammalian cells containing the chimeric protein-encoding DNA or RNA are cultured under conditions appropriate for growth of the cells and expression of the DNA or RNA. Those cells which express the chimeric protein can be identified, using known methods and methods described herein, and the chimeric protein isolated and purified, using known
20 methods and methods also described herein; either with or without amplification of chimeric protein production. Identification can be carried out, for example, through screening genetically modified mammalian cells displaying a phenotype indicative of the presence of DNA or RNA encoding the chimeric protein, such as PCR screening, screening by Southern blot analysis, or screening for the expression of the chimeric protein. Selection of cells
25 having incorporated chimeric protein-encoding DNA may be accomplished by including a selectable marker in the DNA construct and culturing transfected or infected cells containing a selectable marker gene under conditions appropriate for survival of only those cells which express the selectable marker gene. Further amplification of the introduced DNA construct can be effected by culturing genetically modified mammalian cells under conditions
30 appropriate for amplification (e.g., culturing genetically modified mammalian cells containing an amplifiable marker gene in the presence of a concentration of a drug at which only cells containing multiple copies of the amplifiable marker gene can survive).

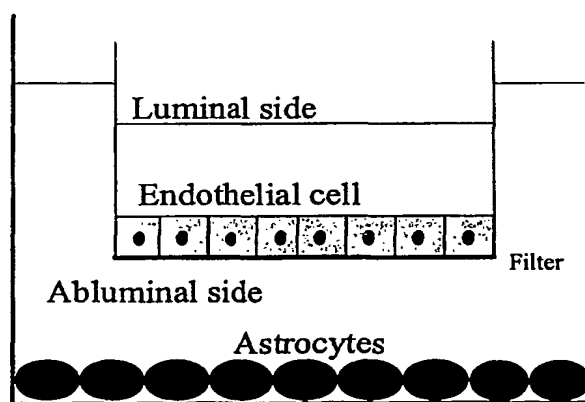
[218] Genetically modified mammalian cells expressing the chimeric protein can be identified, as described herein, by detection of the expression product. For example, mammalian cells expressing chimeric protein in which the carrier is p97 can be identified by a sandwich enzyme immunoassay. The antibodies can be directed toward the LRP portion or the active agent portion.

EXAMPLES

The examples provide exemplary protocols for assessing transcytosis *in vitro* and for characterizing the interaction of p97 and MTF-R receptor modulators or ligands with the p97 receptor or the blood-brain barrier:

Example I : *In vitro* model of the blood-brain barrier.

In vitro model of the BBB



[219] A. **Astrocytes.** Primary cultures of mixed astrocytes were prepared from newborn rat cerebral cortex (Dehouck, *et al.*, *Journal of Controlled Release* (1992)). Briefly, after removing the meninges, the brain tissue was forced gently through a 82 μ m nylon sieve. Astrocytes were plated on six-well microplates at a concentration of 1.2×10^5 cells/ml in 2 ml of optimal culture medium (DMEM) supplemented with 10% fetal heat inactivated calf serum. The medium was changed twice a week.

[220] B. **BBCEC.** Bovine brain capillary endothelial cells (BBCECs) were obtained from Cellial Technologies. The cells were cultured in the presence of DMEM medium

supplemented with 10% (v/v) horse and 10% heat-inactivated calf serum, 2 mM glutamine, 50 µg/ml gentamycin, and 1 ng/ml basic fibroblast growth factor, added every other day.

[221] C. BBB. The *in vitro* model of BBB was established by using a co-culture of BBCECs and astrocytes, basically as described in Dehouck, *et al.*, *Eur. J. Pharm. Sci.*, 3:357-365 (1995); and Cecchelli, *et al.*, *Adv. Drug Deliv. Rev.*, 36:165-178 (1999)). In this model, the luminal side corresponds to the apical or serum facing side of the blood-brain barrier. The abluminal side corresponds to the basolateral side of the BBB, *i.e.*, the side facing the neurons. It is noted that this terminology is used throughout the specification. Prior to cell culture, plate inserts (Millicell-PC 3.0 µm; 30-mm diameter) were coated on the upper side with rat tail collagen. They were then set in the six-multiwell microplates containing the astrocytes prepared as described above, and BBCECs were plated on the upper side of the filters in 2 ml of co-culture medium. This BBCEC medium was changed three times a week. Under these conditions, differentiated BBCECs formed a confluent monolayer 7 days later. Experiments were performed between 5 and 7 days after confluence was reached. The number of cells at confluence was 400 000 cells/4.2 cm² or 90 µg of protein/4.2 cm², as evaluated by a micro-BCA assay from Pierce (Rockford, IL).

[222] D. Sucrose permeability. The permeability coefficient for sucrose was measured to verify the endothelial permeability and the integrity and tightness of the BBCEC monolayers, as previously described, using uncoated filters or coated with endothelial cells. Briefly, the results were plotted as the clearance of [¹⁴C]-sucrose (µl) as a function of time (min). The permeability coefficient (Pe) was calculated as: $1/Pe = (1/PSt - 1/PSf) / \text{filter area (4.2 cm}^2\text{)}$, where PSt is the permeability x surface area of a filter of the co-culture; PSf is the permeability of a filter coated with collagen and with astrocytes plated on the bottom side of the filter.

[223] Brain EC monolayers grown on inserts were transferred to 6-well plates containing 2 ml of Ringer/Hepes per well (basolateral compartment). Ringer/Hepes solution was composed of 150 mM NaCl, 5.2 mM KCl, 2.2 mM CaCl₂, 0.2 mM MgCl₂, 6 mM NaHCO₃, 5 mM Hepes, 2.8 mM Hepes, pH 7.4. In each apical chamber, the culture medium was replaced by Ringer/Hepes containing the labeled [¹⁴C]-sucrose. At different times, inserts were placed into another well. At the end of the experiments, amounts of the radiotracers in the basolateral compartment were measured in a liquid scintillation counter. The permeability coefficient (Pe) for sucrose was calculated as previously described (Dehouck, *et al.*, *J. Neurochem.*, 58:1790-1797 (1992)) using filters coated or noncoated with EC. At the

end of the experiments, amounts of the radiotracers in the basolateral compartment were measured in a liquid scintillation counter. The results were plotted as the clearance of [^{14}C]-sucrose (μl) as a function of time (min). $\text{PSt} = \text{permeability} \times \text{surface area of a filter of the coculture}$; $\text{PSf} = \text{permeability of a filter coated with collagen and astrocytes plated on the bottom side of the filter}$. The permeability coefficient (Pe) was calculated as:

$$1) \text{ Clearance } (\mu\text{l}) = \frac{[\text{C}]_{\text{A}} \times V_{\text{A}}}{[\text{C}]_{\text{L}}} \quad \begin{array}{l} [\text{C}]_{\text{A}} = \text{Abluminal tracer concentration} \\ V_{\text{A}} = \text{Volume of abluminal chamber} \\ [\text{C}]_{\text{L}} = \text{Luminal tracer concentration} \end{array}$$

$$2) 1/\text{Pe} = (1/\text{PSt} - 1/\text{PSf}) / \text{filter area } (4.2 \text{ cm}^2)$$

10 [224] Figure 1 and Figure 2 set out control experiments.

[225] Briefly, in Figure 1, coated or non-coated filters with BBCE cells were transferred to 6-well plates containing 2 ml of Ringer/Hepes per well (basolateral compartment) for 2 hrs at 37°C . In each apical chamber, the culture medium was replaced by 1 ml Ringer-Hepes containing labeled [^{14}C]-sucrose. At different times, inserts were placed into another well.

15 At the end of the experiments, amounts of the radiotracers in the basolateral compartment were measured in a liquid scintillation counter. The difference in the slopes for PSf and PSt demonstrates that the monolayer of cells provides significant resistance to sucrose permeability, thus confirming the presence of tight junctions between cells regardless of pre-incubation.

20 [226] In Figure 2, 1 micromolar p97 protein (provided by Synapse Technologies Inc, Vancouver, Canada) was added to the luminal side to determine its effect on sucrose permeability. Sucrose permeability in the absence of p97 was $1.21 \times 10^{-3} \text{ cm/min}$, whereas in the presence of p97 sucrose permeability was $1.35 \times 10^{-3} \text{ cm/min}$. The results show that there was no significant change in sucrose permeability. A control experiment in the absence
25 of cells demonstrates the relative effectiveness of the BBB model compared to the filter alone.

[227] In Figures 1 and 2, Psf and Pst refer to flow rates through the membrane with and without cells. They are used to calculate the permeability of the membrane with cells. Definitions for Psf and Pst are found in *J. Neurochem.*, 58:1790-97 (1992), the teachings
30 of which are incorporated by reference. The definition of PSt and PSf are simply the slope of the clearance curves for the co-culture and for the control filter respectively. The PS value for the endothelial monolayer alone is defined as PSe where:

$$1/\text{PSe} = 1/\text{PSt} - 1/\text{PSf}$$

Permeability of the endothelial monolayer alone is defined as P_e where:

$P_e = P_{Se}/A$, wherein A is the area of the membrane.

Example II : Binding of p97 with BBCECs and Rat Brain Endothelial Cells

5 [228] B. Binding studies of p97. Binding of p97 was performed with BBCECs and Rat Brain Endothelial Cells that were pre-incubated 2 hrs in Ringer/Hepes to avoid any interference from the astrocytes.

[229] Figure 3 demonstrates the competitive binding of p97 with cold p97, transferrin and lactoferrin. For the binding experiments, cells were incubated for 2 h at 4°C in Ringer/Hepes
10 in the presence of [¹²⁵I]-p97 (25 nM) and increasing concentrations of cold-p97 or high (7.5 micromolar) concentrations of transferrin or lactoferrin. At the end of the incubation, the filters were gently washed at 4°C three times with 4 ml of cold-PBS. Then the associated radioactivity of endothelial cells was determined by removing the membrane of the culture insert and counting it in a gamma counter.

15 [230] The results of Figure 3 demonstrate that [¹²⁵I]-p97 binding to the BBCECs was competitively inhibited by cold-p97 and by lactoferrin, but significantly, transferrin did not block or reduce the binding of [¹²⁵I]-p97 to its receptor on the BBCECs. This data establishes for the first time that [¹²⁵I]-p97 is not binding to the transferrin receptor (Tf-R) as previously hypothesized.

20 **C. Binding of p97 in rat brain endothelial cells.**

[231] As with Figure 3, Figure 4 demonstrates a comparative study, but this time using Rat Brain Endothelial-4 cells (RBE4 supplied commercially by ATCC). RBE4 cells were grown in monolayers in 24 wells plastic tissue culture flasks at 37°C under 5% CO₂ in minimum essential medium Alpha and Ham's F10 (1 :1) supplemented with 10% heat inactivated fetal
25 bovine serum. For p97 binding experiments, RBE4 cells were pre-incubated at 37°C for 2 hrs in Ringer/Hepes. ¹²⁵I-p97 in 200 µl of Ringer/Hepes was added to RBE4 cells for 2 hrs at 4°C in the presence or absence of high concentration of cold-p97, human holo-transferrin or human lactoferrin. After the incubation, the cells were washed 4 times with PBS and the ¹²⁵I-p97 associated with the cells was measured.

[232] Figure 4 demonstrates that cold p97 (10 micromolar) and lactoferrin (10 micromolar) both competitively inhibit [¹²⁵I]-p97 binding to RBE4 cells, whereas transferrin (10 micromolar) does not.

Example III : Interaction of p97 with human brain capillaries.

5 [233] A. **Isolation of human brain capillaries.** Capillaries of the blood-brain barrier were isolated from human brain cortex by a procedure previously described by Dallaire, *et al.*, *J. Biol. Chem.*, 267:22323-22327 (1992) with slight modifications. Human brains were obtained post-mortem. The brain was cleared of meninges, superficial large blood vessels and choroid plexus. All the following procedures were performed at 4°C. The cerebral
10 cortex was homogenized in 5 volumes of Ringer/Hepes solution with a Polytron (Brinkman Instruments, Rexdale, Ontario, Canada). The homogenate was mixed with an equal volume of Dextran T-70 (27g in 100 ml of Ringer/Hepes). The suspension was centrifuged at 25,000 g for 10 min. The pellet was resuspended in 30 ml of Ringer/Hepes and passed through a 250 µm nylon mesh screen. The nylon mesh was rinsed and the filtrate was concentrated by
15 centrifugation at 25,000 g for 10 min. The pellet was resuspended in 30 ml of cold-Ringer/Hepes and passed through a 2.5 cm x 4.0 cm glass beads column [40/60-mesh (0.25 mm) glass beads]. The columns were washed twice with 25 ml of Ringer/Hepes. The glass beads were transferred to a beaker and swirled vigorously (15 min at 4°C) in Ringer/Hepes to separate the microvessels from the beads. The beads were allowed to settle and the
20 supernatant was decanted and kept at 4°C. The beads were swirled for another 15 min in Ringer/Hepes. The supernatants were pooled and the microvessels were collected by centrifugation at 25,000 g for 10 min. Brain capillaries were kept at -80°C until used.

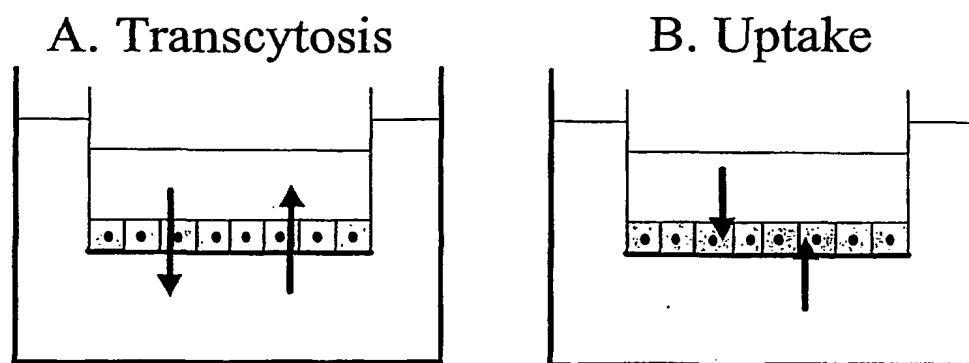
[234] B. **p97 accumulation in human brain capillaries.** A rapid filtration technique was used to measure the accumulation of [¹²⁵I]-p97 in human brain capillaries. Accumulation of
25 [¹²⁵I]-p97 was measured at 37°C for 1 h in isolated human brain capillaries (100 µg/assay). The incubation medium contained [¹²⁵I]-p97 and a final concentration of 100 nM p97 in Ringer/Hepes solution. The accumulation of [¹²⁵I]-p97 was performed in presence or in absence of 5 µM of cold-p97, holo-transferrin or lactoferrin. After incubation, the accumulation was stopped by addition of 1 ml-cold stop solution (150 mM KCl, 0.1% BSA
30 and 5 mM Hepes, pH 7.5). The suspension was filtered under vacuum through a 0.45µM pore size Millipore filter. The filter was rinsed with 8 ml of stop solution, and the radioactivity was counted. Nonspecific binding of the radioactivity to the capillaries was

determined by the addition of the ice-cold stop solution to the capillaries before adding the incubation medium. This value was subtracted from accumulation values obtained following an 1 h incubation. The results were expressed as ng of [125 I]-p97 accumulated per μ g of brain capillaries.

- 5 [235] Figure 5 demonstrates the results of the experiments. Consistent with the findings in Figures 3 and 4, it is found that cold p97 (5 micromolar) or lactoferrin (5 micromolar) significantly inhibits competitive [125 I]-p97 binding to human brain capillary cells, whereas transferrin (5 micromolar) shows no significant competition. This finding confirms that p97 binding to its receptor is not blocked by transferrin.
- 10 [236] Figure 6 is a repeat experiment, performed identically to the experiment of Figure 5, except this time with the additional competition assay for β -amyloid peptide. Evidently, the β -amyloid peptide 1-40 competes with p97 for receptor binding, along with lactoferrin, but not with transferrin. This finding indicates that the receptor responsible for the p97 binding and transport across the BBB is RAGE and/or LRP1.
- 15 [237] Figure 7 shows the results of experiments where the ligands p97, Lf and Tf were heated or not heated prior to the binding study. In all cases, binding experiments were conducted in the transwell apparatus as described previously, with the exception that binding was conducted at either 4°C or at 37°C. For the 37°C trial, a separate experiment was conducted where the ligand was boiled for 30 mins then rapidly cooled prior to
- 20 administration on the transwell plates. Results demonstrate that the heat denatured p97 protein had a significantly lower accumulation in the cell monolayer compared to normal p97; although both forms at 37°C bound to a higher degree than at 4°C. Similarly, heat denatured Lf had significantly lower accumulation in the BBB model cells than its natural counterpart. Transferrin itself had very little accumulation to speak of.
- 25 [238] In Figure 7, it is important to clarify the overall striking difference in accumulation of Lf and p97, as Lf is almost twice as high at the end of the experiment. It must be remembered that p97 is being substantially transcytosed through the BBB model cells and secreted into the abluminal region, whereas Lf is not. This effect leads to the incorrect conclusion that Lf may be taken into the BBB at a higher rate than p97.

Example IV: Transcytosis of p97

Transport assays in BBCEC monolayers



[239] Transcytosis experiments were performed as follows. One insert covered with BBCECs was set into a transwell apparatus containing a six-well microplate with 2 ml of Ringer/Hepes and pre-incubated for 2 h at 37°C. Plates were slowly adjusted to the indicated temperatures (4°C or 37°C). [¹²⁵I]-p97 (250 nM) was added to the upper side of the filter covered with cells. At various times, the insert was transferred to another well to avoid a possible reendocytosis of p97 by the abluminal side of the BBCECs. At the end of experiment, [¹²⁵I]-p97 was assessed in 500 µl of the lower chamber of well by TCA precipitation. p97 was also measured in 50 µl of the lower chamber of the well by Western blots using mAb L235.

[240] Figure 8 demonstrates that p97 transcytosis was significantly higher at 37°C than at 4°C. This result demonstrates that p97 is actively transported in an energy dependent process across this blood-brain barrier model in a temperature dependent fashion, presumably by receptor mediated uptake.

[241] Figure 9 confirms that transcytosis of p97 is also a saturable phenomenon, thus further implicating a specific MTF-receptor protein in this model of the blood-brain barrier. These experiments were conducted as previously described. Measurements of the amount of transcytosis were made at the time points indicated.

[242] The effect of potentially competitive ligands on transcytosis was assessed in a series of experiments. In Figure 10a, transcytosis of ¹²⁵I-p97 was compared in the presence of cold

p97 (5 micromolar), Lf (5 micromolar), and Tf (5 micromolar). At these concentrations, only the cold p97 successfully reduced transcytosis of the labelled p97. In Figure 10b, β -amyloid protein (5 micromolar) also failed to slow or reduce transcytosis of labelled p97. Higher amounts of ligands do interfere with p97 transcytosis (data not shown). In Figure 10c, RAP, a known polypeptide inhibitor of the LDL-Receptor family was applied to the cells (25 micrograms/ml). RAP significantly inhibited the transcytosis of p97, thus directly implicating the LDL-receptor family, especially LRP1 as the MTf-R.

Example V. Accumulation and transcytosis of p97 in brain.

[243] A. **Brain uptake and in situ brain perfusion.** To measure the brain uptake of [125 I]-p97, mice were each given approximately 4 pmol of [125 I]-p97, [125 I]-BSA or human [125 I]-holo-transferrin in 200 μ l of injection solution through the jugular vein. After 1 hour, animals were sacrificed and perfused with buffer via cardiac aorta. The serum and brain samples were collected and the levels of radioactivity were measured. In situ brain perfusion was performed as previously described (Dagenais, C., Rousselle, C., Pollack, G.M. & Scherrmann, J.M. *J.Cereb. Blood Flow Metab.* 20: 381-386 (2000)). Briefly, the right hemisphere of the brain was perfused with 10 nM of [125 I]-p97 or [125 I]-holotransferrin in Krebs-bicarbonate buffer (pH 7.4 with 95 % O₂ and 5 % CO₂ at a flow rate of 2.5 ml/min for 10 min) via a catheter inserted in the right common carotid artery following ligation of the external branch. Mice were decapitated to terminate perfusion and the right hemisphere was isolated on ice before subjected to capillary depletion (Triguero, D., Buciak, J. & Pardridge, W.M. *J Neurochem.*, 54: 1882-1888 (1990)). Aliquots of homogenates, supernatants, pellets and perfusates were taken to measure their contents in [125 I]-proteins by TCA precipitation and to evaluate their apparent V_D .

[244] B. **Iodination of proteins.** p97, bovine holo-transferrin and bovine lactoferrin were iodinated with standard procedures using iodo-beads from Sigma. Bovine holo-transferrin and bovine lactoferrin were diluted in 0.1M phosphate buffer, pH 6.5 (PB). p97 obtained from Synapse Technologies in neutralized citrate at pH 7.0 was dialyzed against this PB. Two iodo-beads were used for each protein. These beads were washed twice with 3 ml of PB on a Whatman filter and resuspended in 60 μ l of PB. 125 I (1 mCi) from Amersham-Pharmacia biotech was added to the bead suspension for 5 min at room temperature. The iodination for each protein was initiated by the addition of 100 μ g (80-100 μ l). After an incubation of 10 min at room temperature. The supernatants were applied on a desalting column prepacked with 5 ml of cross-linked dextran from Pierce and 125 I-proteins were eluted

with 10 ml of PBS. Fractions of 0.5 ml were collected, and the radioactivity in 5 μ l of each fraction was measured. Fractions corresponding to 125 I-proteins were pooled and dialyzed against Ringer/Hepes, pH 7.4. The efficiency of radiolabeling was between $0.6-1 \times 10^8$ cpm/100 μ g of protein.

- 5 [245] **Transcytosis and binding experiments.** One insert covered with BBCECs was set into a six-well microplate with 2 ml of Ringer-Hepes and was pre-incubated for 2 h at 37 °C. [125 I]-p97 was then added to the upper side of the insert. At various times, the insert was sequentially transferred into a fresh well to avoid possible reendocytosis of p97 by the abluminal side of the BBCECs. At the end of the experiment, [125 I]-p97 was quantitated in 10 500 μ l of the lower chamber of each well by TCA precipitation. We also measured p97 in 50 μ l of the lower chamber of each well by SDS-PAGE according to the method of Laemmli (Laemmli, U.K. *Nature*; 227: 680-685 (1970)). Proteins were separated on 7.5% acrylamide gels, stained with Coomassie Blue, dried and analysed by densitometry. For the binding 15 cellular membranes as perviously described (Descamps, L., Dehouck, M.P., Torpier, G. & Cecchelli, R. *Am. J. Physiol.* 270: H1149-H1158 (1996)). After 2 hrs at 40C, ECs were gently washed, and the [125 I]-p97 attached to the ECs was quantified in a liquid scintillation counter.

- [246] **p97 accumulation in human brain capillaries.** Human brain capillaries were 20 isolated by a procedure previously described (see Dallaire, L., Tremblay, L. & Beliveau, R. *Biochem. J.* 276: 745-752 (1991), Demeule, M. et al. *Int. J. Cancer.* 93: 62-66 (2001)). [125 I]-p97 was incubated with capillaries (100 μ g) in Ringer/Hepes solution in the presence or absence of unlabelled p97, holo-transferrin or lactoferrin. The uptake was stopped by the addition of ice-cold stop solution (150 mM KCl, 0.1% bovine serum albumin (BSA) and 5 25 mM Hepes, pH 7.5) and the suspension was filtered under vacuum through a 0.45 μ m pore size Millipore filter. The filter was rinsed with 8 ml of stop solution, and the radioactivity was assayed. Nonspecific binding of radioactivity to the capillaries was determined by addition of the ice-cold stop solution to the capillaries before adding the incubation medium. This value was subtracted from the values obtained following a 1 h incubation.

30 **Example VI. Exemplary Methods.**

[247] **Cell culture.** Cells were grown in monolayer at 37°C under 5% CO₂ in DMEM supplemented with 10% fetal heat inactivated calf serum (normal astrocytes); DMEM high glucose, 1mM sodium pyruvate supplemented with 10% calf serum (CTX); DMEM high

glucose supplemented with 10% calf serum (RG2); RPMI-1640 supplemented with 10% calf serum and 2 mM glutamine (CNS-1); Ham's F12 supplemented with 10% calf serum (C6); MEM, 1mM sodium pyruvate supplemented with 10% calf serum (U-87, U-138).

5 [248] Uptake of [125 I]-p97. Cells were grown in monolayer in six-multiwell microplates at 37°C under 5% CO₂. Uptake of [125 I]-p97 was measured at 37°C for 2 h in astrocytes and astrocytomas. The incubation medium contained [125 I]-p97 and a final concentration of 50 nM P97 in Ringer/Hepes solution. The uptake of [125 I]-p97 was performed in presence or in absence of 5 μ M cold-P97. After incubation, the cell monolayer was wash three times with cold Ringer/Hepes solution. Triton X-100 0.1% was added and the [125 I]-p97 uptake was
10 assessed in the Triton X-100 soluble fraction by TCA precipitation.

[249] RNA extraction. Cells in six-wells plate or in 75 cm² plastic tissue culture flasks were grown at 37°C under 5% CO₂ with optimal culture medium to 80-90% confluence. Total cellular RNA was preserved in Trizol (Gibco BRL, Burlington, ON). The solution are frozen at -80 oC until extraction. The solution was defroze and mixed with 5:1 chloroform for
15 3 minutes at room temperature. The suspension was centrifugated at 12000 g at 4oC. The clear supernatant was collected then mixed with 1:1 of isopropanol for 15 minutes at room temperature. The mix was centrifugated at 12000 x g for 10 min at 4oC. The pellets was washed with 70% ethanol and dried before resuspended in RNase Free H₂O.

[250] RT-PCR. RT-PCR was performed for members of the LDL-R family: LRP, LRP1B, megalin, LDL, VLDL, LRP8, LR11, RAP, LR3, cubulin and P97. DNA (cDNA) synthesis
20 was performed with 1 (g of total RNA using a cDNA one step synthesis kit (Invitrogen,USA) following the manufacturer's protocol. (1x of reaction mix, RNA 1(g, 0.2(M of both primers, 1(l of RT/Platinum Tag mix). The cDNA generated was amplified using primers produced with MacVector 7.0 (Oxford molecular Ltd, Oxford, UK). All the subsequent assays were
25 then performed under conditions that produced amplifications of cDNA within a linear range. RT inverse-transcription was performed at 50oC for 30 min. PCR amplification for 35 cycles for all was performed as follows: denaturation at 94°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 30 s. Finalisation stage was performed at 72oC for 30 min. Tubes containing all the ingredients except templates were included in all runs and served as
30 negative controls. The amplified PCR products were electrophoresed on a 2% agarose gel in TAE (40 mM Tris, 360 mM acetic acid, 1 mM EDTA, 12.5 fM Ethium bromide) and were visualized under ultraviolet light followed by densitometric analysis.

[251] **Western blot.** Cell lysates (25 µg of protein) were subjected to SDS-PAGE and electroblotted onto PVDF membranes. Membranes were blocked over night at 4°C in 5% non fat dry milk in TBS (NaCl 125 mM, Tris 20 mM, pH 7,5) with 0.1% Tween. Successive incubations with proper primary antibody and horseradish peroxidase-conjugated secondary antibody were carried out for 60 min at room temperature. All incubations with antibodies were done after 3x15 minutes washes with TBS-Tween 0.1%. The immunoreactive proteins were detected using the ECL system (Amersham-Pharmacia, Baie d'Urfé, Que).

[252] **Ligand-binding.** Briefly, cells were incubated with [¹²⁵I]-p97 in Ringer/Hepes solution, in presence or in absence of 5 µM cold-P97 for 1 h at 4°C. After incubation, the cell monolayer was washed three times with cold Ringer/Hepes solution. After the ligand binding at 4°C, cell monolayer was washed three times with cold Ringer/Hepes solution and was incubated with DSS (Disuccinimidyl suberate) or Lomant's reagent DSP (Dithiobis(succinimidyl propionate)) from Pierce. After the cross-link, cells were lysed and proteins were separated by SDS-PAGE electrophoresis. Gels were fixed, dried and exposed to Kodak films at -80°C for about 3 weeks before developing. The cross-link was performed exactly as the manufacturer's protocol.

[253] **BIACore analysis.** MAb L235 was covalently coupled to a CM5 sensor chip via primary amine groups using the N-hydroxysuccinimide (NHS) /N-ethyl-N'-(dimethylaminopropyl)carbodiimide (EDC) coupling agent as previously described (Johnsson, B., Lofas, S., & Lindquist, G. *Anal. Biochem.* 198: 268-277 (1991)).

[254] Briefly, the carboxymethylated dextran was first activated with 50 µl of NHS/EDC (50 mM/200 mM) at a flow rate of 5 µl/min. The mAb L235 (5 µg) in 10 mM acetate buffer, pH 4.0 was then injected and the unreacted NHS-esters were deactivated with 35 µl of 1 M ethanolamine hydrochloride, pH 8.5. Approximately 8000 to 10000 relative units of mAb 235 were immobilized on the sensor chip surface. The Ringer/Hepes buffer was used as the eluent buffer to monitor the signal plasmon resonance (SPR). p97 diluted in the same eluent buffer was boiled for various lengths of time, cooled to room temperature and injected onto the sensor chip surface. The SPR obtained was compared to that of unboiled p97.

[255] The brain uptake of human [¹²⁵I]-p97 in mice, one hour after i.v. injection was evaluated and compared to that obtained for [¹²⁵I]-BSA or human [¹²⁵I]-transferrin (Fig. 11a). The brain/serum ratio for p97, BSA and holo-transferrin is respectively 0.025, 0.002 and 0.008 indicating a higher brain accumulation for p97. To determine whether this

observation is related to a greater brain penetration, we measured the apparent volume of distribution (V_D) of p97 and transferrin by *in situ* brain perfusion in mice (Fig. 11b). After a 10 min perfusion, the apparent V_D for both proteins was calculated for the whole brain homogenates as well as for brain capillaries and brain parenchyma. Under these conditions the apparent V_D of transferrin in the brain parenchyma is 2.4 ml/100g which is slightly higher than the brain V_D for the vascular marker [^{14}C]-inulin at 1.7 ml/100g (data not shown). Importantly, the apparent V_D of p97 in the brain parenchyma is 17.2 ml/100g, 8.8-fold higher than for transferrin, indicating a greater passage through brain capillaries. To further investigate the transport of p97 across the BBB, the passage of [^{125}I]-p97 across an *in vitro* model of the BBB was measured at 37 °C and at 4 °C. (Fig. 11c). A dramatic reduction in the transport from the apical to the basolateral surface of BBCEC monolayers of [^{125}I]-p97 is observed at 4 °C, indicating that the transcytosis of p97 requires an active mechanism.

[256] Transcytosis of [^{125}I]-p97 at 37 °C was measured both in the apical-to-basolateral direction and in the basolateral-to-apical direction across BBCEC monolayers to ascertain any vectorial transport of p97 (Fig. 11d). This figure demonstrates that p97 transport in the BBCEC model is highly vectorial. In this experiment, [^{125}I]-p97 (25 nM) was added to the luminal or abluminal side of the BBCECs. After 2 hours of incubation at 37°C, the amount on the opposing side of the membrane was measured. Results demonstrate that p97 transport is substantially directed from the luminal to the abluminal side, corresponding *in vivo* to the delivery of p97 from the blood/serum face of brain capillaries (*i.e.*, inside the capillaries) to the neural cells of the brain. After 2 hrs, [^{125}I]-p97 transport is about 3-fold higher when measured in the apical-to-basolateral direction, indicating a substantial preferential transport of p97 towards the brain.

Localization and saturability of the p97 binding activity.

[257] To assess the presence and extent of intracellular p97 binding sites, BBCECs were treated with saponin (Fig. 12a). The saponin permeabilization of ECs increased the amount of [^{125}I]-p97 associated with BBCECs 4-fold. Moreover, the binding of [^{125}I]-p97 after saponin treatment decreased in the presence of unlabelled p97 (Fig. 12b). A 200-fold molar excess of unlabelled p97 inhibited radiolabel binding by approximately 50%, showing that much of the interaction of p97 with ECs is saturable.

[258] Values for specific p97 binding were calculated by subtracting the non-specific binding of p97 measured in the presence of a high concentration of unlabelled p97 and are expressed in a Scatchard plot (Fig. 12c). Analysis of this plot is consistent with a single-binding site for p97 with a K_d of about 1 μ M and 4×10^6 sites/cell.

- 5 [259] Comparison of p97, transferrin and lactoferrin binding on BBCEC monolayers.

	K_d (nM)	Number of binding sites/cells
P97 (+saponin)	1400	6 000 000
Transferrin (+saponin)	11	35 000
Lactoferrin (-saponin)		
Site 1	35	35 000
Site 2	1900	900 000

[260] In the above table, p97 results from figure 12 (K_d and number of binding sites/cells) are compared to published values for transferrin and lactoferrin (see Descamps et al., Am. J. Physiol. 270:H1149-H1158, 1996; Fillebeen et al., J. Biol. Chem. 274 :7011-7017, 1999).

10 **Efficiency of p97 transcytosis**

[261] The efficiency of p97 transcytosis was assessed by comparing the passage of both p97 and bovine holo-transferrin under identical conditions (Fig. 13a). Transport of p97 from the apical to the basolateral surface of ECs is much higher than for transferrin at 37°C (Fig. 13a). Heat-denaturation reduced the passage of both p97 and holo-transferrin through the BBCEC monolayers, indicating that their transcytosis is conformation-dependent. As p97 is resistant to heat denaturation in Ringer/Hepes solution (Fig. 13b), it was necessary to determine the denaturing conditions. The conformation of the protein was assessed using the biological interaction analysis in real-time between p97 and the monoclonal antibody (mAb) L235, which recognizes a conformational epitope on p97 since no enzymatic activity has yet been defined for this protein. For this analytical approach, mAb L235 was immobilized on the surface of a sensor chip and exposed to native p97 as well as to p97 which had been boiled for 5, 10, 20 or 30 min. The surface plasmon resonance signal of native proteins, the accumulation of p97 in BBCE cells is 5.7 μ g/cm² whereas no significant accumulation is

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observed for bovine transferrin. These results show that the p97 transport system has much greater capacity than has the transferrin transport system.

p97 stability and BBCEC monolayer integrity following transendothelial transport

5 [262] To examine p97 integrity after transcytosis at 37 °C and 4 °C, 50 µl of the lower compartments of the wells were recovered after 30, 60, 80 and 120 min. Proteins were then separated by SDS-PAGE and visualized by gel staining (Fig. 14a). Time-dependent transcytosis of recombinant p97 is observed, with no apparent degradation. Transcytosis of this protein is much higher when the experiment is performed at 37 °C than at 4 °C. The low
10 molecular weight proteins observed at 30 min are only serum proteins remaining in the assay. Furthermore, the gels were scanned and the amount of p97 that passed through the BBCEC monolayers was evaluated using known quantities of p97 (Fig. 14b). The total amount of intact p97 after transendothelial transcytosis is 35 µg/cm², very similar to the amount shown in Fig. 13a after TCA precipitation, indicating that the iodination of p97 does not interfere
15 with its transcytosis. Since p97 is transported much faster than is transferrin, the permeability to [¹⁴C]-sucrose was measured in the presence of a high concentration of p97 (Fig. 14c). No significant increase in the clearance of sucrose is detectable in the presence of p97. In addition, the permeability coefficient (Pe) for sucrose in the presence of p97 is $1.04 \pm 0.15 \times 10^{-3}$ cm/min, not significantly different from the value of $1.07 \pm 0.19 \times 10^{-3}$ cm/min measured
20 in the absence of p97 (Fig. 14d). These data indicate that the rapid passage of p97 is unrelated to changes in the integrity of BBCEC monolayers.

Effect of p97 and transferrin on [¹²⁵I]-p97 transcytosis

[263] To establish whether this p97 transport is saturable, and whether it involved the
25 transferrin receptor, apical-to-basal transport of [¹²⁵I]-p97 across BBCEC monolayers was measured in the presence of a 200-fold molar excess of p97, bovine holo-transferrin or human holo-transferrin (Fig. 15). An excess of unlabelled p97 reduced the transport of [¹²⁵I]-p97 by 69% (Fig. 15a) whereas the presence of either bovine or human holo-transferrin had no impact (Fig. 15b). This indicates that p97 transcytosis is a saturable process that does not
30 employ the transferrin receptor. This assumption is supported by the fact that mAb OX-26, which binds to the transferrin receptor, does not significantly reduce p97 transcytosis as compared to transcytosis measured in the presence of nonspecific IgGs (Fig. 15c).

Identification of LRP as a potential receptor for p97

[264] We also assessed the uptake of [125 I]-p97 into isolated human brain capillaries incubated for 1 h at 37 °C (Fig. 16a). A 50-fold molar excess of unlabelled p97 inhibited the uptake of [125 I]-p97 by 60%. Human lactoferrin caused a similar inhibition of [125 I]-p97 uptake whereas human holo-transferrin had no effect. These results indicate that LRP, which binds and transports lactoferrin across BBCEC monolayers is also involved in the uptake of [125 I]-p97 into brain capillaries and in the transcytosis of p97.

[265] To further investigate the role of LRP in the transport of p97, transcytosis experiments were performed in the presence of the receptor-associated protein (RAP), a protein chaperone that regulates LRP (Fig. 16b). Recombinant RAP (25 µg/ml) reduced the initial rate of [125 I]-p97 transport across BBCEC monolayers by more than 50%. In addition, the transcytosis of bovine [125 I]-lactoferrin is inhibited by more than 75% by a 200-fold molar excess of unlabelled p97 (Fig. 16c).

[266] Figure 17 further illustrates the effect of LRP ligands RAP, Aprotinin and BSA on p97 transcytosis and the effect of p97 on lactoferrin transcytosis. The inhibition by RAP but not BSA distinguishes the p97 from megalin, the chylomicron receptor found in the liver, and the receptor for advanced glycation end products which can be found on lung endothelial cells, neurons, astrocytes, and glomeruli.

Time course of p97 uptake in BBCE.

[267] The time course for the internalization of p97 is illustrated in Figure 18 which depicts the movement of p97 at 30 minutes and 60 minutes and its accumulation in early endosome. Figure 19 illustrates the conditions for studying the rate of p97 internalization and transcytosis in the BBB model. Figure 20 shows the results of such a study. Transcytosis is fast as in 10 minutes, 80% of membrane bound p97 has transcytosed.

Pathway for p97 endocytosis.

[268] Upon binding to a LRP to a growing brain capillary endothelial cell, LDL is classically internalized by a clathrin dependent pathway wherein the LDL is directed toward lysosomes and degraded so as to provide cholesterol to the growing cell. This pathway is sensitive to filipin. In differentiated BCECs, the LDL is transcytosed. Evidence indicates the same receptor is involved in both pathways. (see Dehouck et al., *J. of Cell Biology* 138(4) 877-889 (1997).

[269] The possibility that p97 endocytosis involved a clathrin-dependent mechanism or a clathrin independent mechanism (i.e., caveolae) was examined in BBCE. p97 was found to co-localize with clathrin much more similarly than with caveolin (data not shown).

Transcellular localization of p97 and clathrin was examined in BBCE as the p97 moved from the luminal to abluminal side of the BBB model (Figure 21). The data are indicative of a co-migration of p97 and clathrin. As a control for the effect of the Alexa label, the behavior of p97 and p97-alexa were compared. (data not shown). Although p97-alexa also labeled other vesicular structures, p97 and p97-alexa had a similar localization indicating that the label did not change the behavior of p97.

[270] Following endocytosis, the transport fate of p97 depends upon the cell type. As shown in Figure 22, p97 may be transported across the cell as occurs with brain endothelial cells lining the brain capillaries or else it may be transported to a lysosome. In the first instance, p97 is useful as a means of delivering therapeutic agents such as p97 therapeutic agent conjugates across the blood brain barrier. In the second instance, p97 is useful as a means of delivering therapeutic agents to the intracellular compartment, particularly the lysosome. In this instance, conjugates with enzymes (e.g., an enzyme deficient in a patient with a lysosomal storage disease) are particularly of interest.

LRP Ligands and receptor family expression.

[271] Figure 23 illustrates an LRP receptor α and β subunits with respect to the cell membrane and some LRP ligands. Figure 25 presents the relative amounts of LRP/LRP1B proteins in various cell types, including astrocytomas, normal astrocytes, and brain capillaries. As a contrast, the distribution of megalin among astrocytes and astrocytomas is presented in Figure 26. As shown in figures 27 and 28, the expression of LRP1B in astrocytes and astrocytomas as determined by (RT-PCR) is increased in the astrocytomas over the astrocytes. This result is result is consistent with the greater uptake of the labeled p97 by the astrocytomas. By way of contrast, and in confirmation that megalin is not involved in the p97 uptake, the pattern of megalin expression among these cells is not at all similar to the uptake pattern for p97 for these cells (see Figure 26). Figure 28 more clearly illustrates the correlation between p97 uptake and LRP1B expression.

[272] Figure 32 shows the expression of members of the LDL receptor family by RT-PCR. In glioblastoma cell line U87 LRP, LRP1b, LDL, and LRP8 are highly expressed. Cubilin

and RAP are expressed to a much lesser extent. In human capillaries, LRP1B, megalin, LDL, LRP8, and LRP are highly expressed as compared to cubilin.

Association of p97 with LRP/LRP1B

[273] Figure 29 shows that LRP/LRP1B migrates as a high molecular weight dissociable complex in the presence of p97, indicating the association of the two molecules. Exposing the complex to reducing conditions such as β -mercaptoethanol induces the release of p97 from the high molecular weight complex (see Figure 30). p97 similarly forms high molecular weight complexes upon contact with glioblastomas and human brain capillaries (see Figure 31).

Effect of p97 on cell expression of members of LDL-receptor family in human glioblastoma.

[274] To examine the ability of p97 to be taken up by astrocytoma cells and astrocytes, cells of each type were incubated with ^{125}I p97. As shown in Figure, 24, the specific uptake of p97 in such cells was greatly increased in the astrocytoma cells over the astrocytes. As shown in figure 24, astrocytoma cells take up p97 to a much greater extent than astrocytes. This finding indicates that p97 conjugates to anticancer agents would be particularly useful in the treatment of gliomas. Figure 34 shows the effect of p97 and RAP treatments on LRP and LRP1B expression in U87 cells. While p97 does induce the expression of LRP1B, it has no discernable effect on the morphology of the U-87 cells (see figure 33). Figure 35, shows the dose response effect of p97 for LRP1Bb, LRP, LDL-R, Megalin, and Cubilin as measured by RT-PR in U-87 cells. p97 induces the expression of the LRP1B receptor and the Cubilin receptor, but not the LRP or Megalin receptor. RAP appears to antagonize the effect of p97 on those receptors. p97 also appears to induce its own expression as shown by RT-PCR methods (see Figure 36). Figures 37 and 38 summarize the quantitated effects of p97 and RAP on the expression of LRP1B, LRP, LDL-R, Cubilin, p97, and Megalin in the glioblastoma U87 cell line as determined by RT-PCR.

Effect of p97 on Fibroblast Cells.

[276] The fibroblast cell line MG1391 was used to assess the potential of p97 and other LRP ligand conjugates as carriers for transcytosis or endocytosis in fibroblasts. Figure 39 shows the expression of members of the LDL receptor family in MG1391 cells. LRP, LDL, and to a lesser extent, LRP1B and Cubilin are expressed as measured by RT-PCR. Furthermore, p97 particularly induces the expression of the LRP1B as shown in Figures 40 and 41.

Expression of LDL receptor Family members in human endothelial cells and BBCE cells.

[277] As shown in Figure 42, LRP, LRP1B, and LDL-R as well as LRP8 are highly expressed in human endothelial cells. The expression of LDL receptor family members in the absence of astrocytes is largely that of LRP5. However, the presence of astrocytes induces the expression of LRP1B and LRP8 in the BBCE cells (see Figure 43).

[278] The above examples show that the brain p97 uptake, in vivo, is much higher than that of other proteins such as BSA and transferrin. The in vitro model of the BBB used here to characterize the transcytosis of p97 has been used previously for such proteins as transferrin, lactoferrin, low density lipoproteins and insulin (see Dehouck, B. et al. *J. Cell. Biol.* 138: 877-889 (1997), Fillebeen, C. et al. *J. Biol. Chem.* 274: 7011-7017 (1999); Descamps, L., Dehouck, M.P., Torpier, G. & Cecchelli, R. *Am. J. Physiol.* 270: H1149-H1158 (1996)); Frank, H.J., Pardridge, W.M., Morris, W.L., Rosenfeld, R.G. & Choi, T.B. *Diabetes* 35: 654-661 (1986)). As was seen with these proteins, transendothelial transport of p97 requires energy and is concentration-dependent, indicating a receptor-mediated endocytosis mechanism for p97. In addition, preferential transport of p97 from the apical to the basolateral surface of BBCECs is observed with no detectable degradation of p97. The conformation of p97 also seems to be very important for its transcytosis because heat-denaturation considerably reduced the transendothelial transport of this protein. Thus, the in vitro results strongly confirm and support the in vivo observations on high p97 uptake in the brain.

[279] The results show the presence of a low affinity receptor for p97 with a high capacity. Since all the experiments comparing bovine transferrin with human p97 are performed in a heterologous system, we can expect that the binding constant for the p97 receptor would be even greater in a human homologous system. It has been postulated that p97 is an alternate ligand for the transferrin receptor because p97 shares many properties with human transferrin and because the transferrin receptor has been detected in the same tissues as p97. However, our results strongly support that a different mechanism than that involving the transferrin receptor. First, the transcytosis, binding and accumulation of p97 are much higher than those for transferrin indicating that the p97 receptor has a much higher capacity and lower affinity than those previously reported for the transferrin receptor. Second, the transcytosis of p97 is unaffected by either bovine or human transferrin, indicating that p97 does not compete with transferrin for its receptor. Third, the mAb OX-26 directed against the transferrin receptor,

which was previously shown to inhibit the uptake of transferrin, has no effect on p97 transport. In addition to the transcytosis experiments using BBCEC monolayers, the competition of [125I]-p97 uptake by unlabelled p97 in isolated human brain capillaries confirmed the presence of a receptor for p97. Moreover, lactoferrin competed [125I]-p97 uptake efficiently, better than transferrin or any other tested proteins, indicating that lactoferrin and p97 share a receptor. The receptor for lactoferrin transcytosis in brain ECs is LRP 6, a member of the large LDL-receptor family. (see Bu, G. & Rennke, S. J. *Biol. Chem.* 271: 22218-2224 (1996)). To further investigate whether LRP could be involved in p97 transcytosis, experiments were performed with BBCEC monolayers in the presence of RAP, a protein which inhibits the binding of ligand to members of the LDL-receptor family (see Bu, G. & Rennke, S. J. *Biol. Chem.* 271: 22218-2224 (1996); Willnow, T.E, Goldstein, J.L., Orth, K., Brown, M.S. & Herz, J. *J. Biol. Chem.* 267: 26172-26180 (1992); Bu, G. & Schwartz, A.L. *Trends Cell Biol.* 8: 272-276 (1998); and Herz, J. & Strickland, D.K. *J. Clin. Invest.* 108: 779-784 (2001)).

[280] Known members of this family also include LDL-R, LRP1B, megalin, VLDL-R, apoE-receptor 2 and the mosaic LDLR-related protein (LR11) (see Hussain, M.M. *Front. Biosci.* 6: D417-D428 (2001); and Liu, C.X., Li, Y., Obermoeller-McCormick, L.M., Schwartz, A.L. & Bu, G. *J. Biol. Chem.* 276: 28889-28896 (2001)). Among these receptors, megalin, is also known to bind lactoferrin (see Hussain, M.M. *Front. Biosci.* 6: D417-D428 (2001); and Willnow TE. *Biol. Chem.* 379: 1025-1031 (1998)). However, megalin is mainly expressed in the kidney whereas the major site of LRP expression is in brain. Thus, the diminution of p97 transcytosis by RAP and the inhibition of lactoferrin transcytosis by p97 also indicates that LRP transports p97 across BBCECs.

[281] The concept of using receptor-mediated endocytosis to deliver peptides into the brain was initially described with the findings on the transendothelial transport of insulin across the blood brain barrier (BBB). Subsequent studies demonstrated that a neuropeptide could be delivered into the CNS using receptor-mediated endocytosis by targeting the transferrin receptor with the mAb OX-26 (see Bickel, U., Yoshikawa, T. & Pardridge, W.M. *Adv. Drug Deliv. Rev.* 46: 247-279 (2001) and Pardridge, W.M., Buciak, J.L. & Friden, P.M. *J. Pharmacol. Exp. Ther.* 259: 66-70 (1991)). The development of chimeric proteins containing this mAb, specific linkers and a neurotropic peptide has permitted delivery into the brain of significant levels of this peptide (see Bickel, U., Yoshikawa, T. & Pardridge, W.M. *Adv. Drug Deliv. Rev.* 46: 247-279 (2001); Pardridge, W.M., Wu, D., & Sakane, T. *Pharm. Res.*

15: 576-582 (1998); and Zhang, Y. & Pardridge, W.M. *Brain Res.* 889: 49-56 (2001)). In addition, the transendothelial transport of mAb OX-26 was also reported in these studies to be similar to the transport of human transferrin across the BBB. Our results show that p97 passes across the blood brain barrier (BBB) at least as well as does OX-26. Another

5 advantage of using p97 is its very low concentration in the serum (100 000-fold lower than transferrin) (see Jefferies, W.A. et al. *Brain Res.* 712: 122-126 (1996), and Kim, D.K. et al. *S Neuropsychopharmacology* 25: 84-90 (2001)), which indicates that it would deliver p97-conjugate(s) directly into the CNS.

[282] The results show that intact p97 can cross brain ECs without affecting the integrity of
10 the BBB and with a much higher rate than is seen with transferrin. The inhibition of p97 transcytosis by RAP in BBCEC monolayers and the competition of p97 uptake in brain capillaries by human lactoferrin show that LRP, a member of the LDL-R family, is involved in the transendothelial transport of p97. The results indicate that p97 and, more generally, ligands of the LRP and LRP1B receptors are preferred carriers for conjugation with active
15 agents and preferred modulators for the transport of such conjugates via the LRP or LRP1B receptor.

Each publication, patent application, patent, and other reference cited in any part of the specification is incorporated herein by reference in its entirety to the extent that it is not inconsistent with the present disclosure.

20 Based on the invention and examples disclosed herein, those skilled in the art will be able to develop other embodiments of the invention. The examples are not intended to limit the scope of the claims set out below in any way. Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of the
25 teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

WHAT IS CLAIMED IS:

- 1 1. A method for identifying a compound that modulates the transcytosis
2 or endocytosis of melanotransferrin or a melanotransferrin conjugate with an active agent,
3 said method comprising:
4 contacting melanotransferrin ("MTf") and said MTf-receptor ("MTf-R") with
5 said compound; and
6 determining the functional effect of said compound.
- 1 2. The method of claim 1 wherein said compound has neurological
2 activity.
- 1 3. The method of claim 2 wherein the neurological activity is treatment,
2 prophylaxis or diagnosis of a neurological disorder.
- 1 4. The use of claim 2 wherein the neurological activity is to reduce an
2 undesirable side-effect of a therapeutic agent.
- 1 5. The use of claim 3 wherein the neurological activity is modulation of
2 the uptake of melanotransferrin conjugated therapeutic agents into the brain.
- 1 6. The method of claim 1 wherein the method is a high throughput
2 screening assay.
- 1 7. A method of modulating a melanotransferrin receptor ("MTf-R"), said
2 method comprising: contacting said MTf-R with a compound identified using the method of
3 claim 1.
- 1 8. A method of treating a neurological disorder in a patient, said method
2 comprising administering to said patient a therapeutically effective amount of a compound
3 identified using the method of claim 1.
- 1 9 A method of claim 1, wherein the MTf-R is LRP or LRP1B.
- 1 10 The method of claim 1, wherein the compound is a endogenous human
2 MTf-R ligand.
- 1 11. The method of claim 1, wherein the MTf-R is human.

1 12. A method of claim 1, wherein the compound is lactoferrin or RAP.

1 13. A method for increasing the uptake of a melanotransferrin conjugated
2 therapeutic agent into the brain of a patient, said method comprising administering a
3 modulator of MTf-R biological activity and said melanotransferrin conjugated therapeutic
4 agent.

1 14. The method of claim 13 wherein said modulator of MTf-R biological
2 activity and said melanotransferrin conjugated therapeutic agent are administered
3 contemporaneously.

1 15. The method of claim 9 wherein said modulator of MTf-R biological
2 activity and said melanotransferrin conjugated therapeutic agent are administered
3 sequentially.

1 16. A method of reducing the uptake of a melanotransferrin conjugated
2 therapeutic agent into the brain of a patient, said method comprising administering a
3 modulator of MTf-R biological activity either contemporaneously or sequentially with a
4 melanotransferrin conjugated therapeutic agent.

1 17. The method of claim 13 wherein the modulator is first identified
2 according to the method of claim 1.

1 18. A modulator of MTf-R biological activity, said modulator identified
2 using the method of claim 1.

1 19. The modulator of claim 18 wherein said modulator is useful for
2 reducing a neurological side-effect of a therapeutic agent.

1 20. A method of identifying a compound that modulates
2 melanotransferrin-mediated ("MTf-mediated) iron uptake, said method comprising:
3 contacting a cell expressing MTf on its surface with said compound in the presence of MTf
4 bound to iron ("halo-MTf") and in the absence of transferrin; and determining the amount of
5 iron uptake into said cell.

1 21. The method of claim 20, wherein said compound increases the amount
2 of iron uptake into said cell.

- 1 22. The method of claim 20, wherein said compound decreases the amount
2 of iron uptake into said cell.
- 1 23. The method of claim 20, wherein MTf-R is LRP1 or LRP1B.
- 1 24. The method of claim 23, wherein the LRP1B is human.
- 1 25. A conjugate of an LRP1B receptor ligand with an active agent,
2 wherein the ligand is not selected from the group comprising p97, lactoferrin, transferrin
3 RAP, or fragments thereof.
- 1 26. A conjugate of claim 25, wherein the conjugate is a fusion protein.
- 1 27. A conjugate of claim 25, wherein the active agent is an enzyme.
- 1 28. A conjugate of claim 27, wherein the enzyme is an enzyme deficient in
2 a lysosomal storage disease.

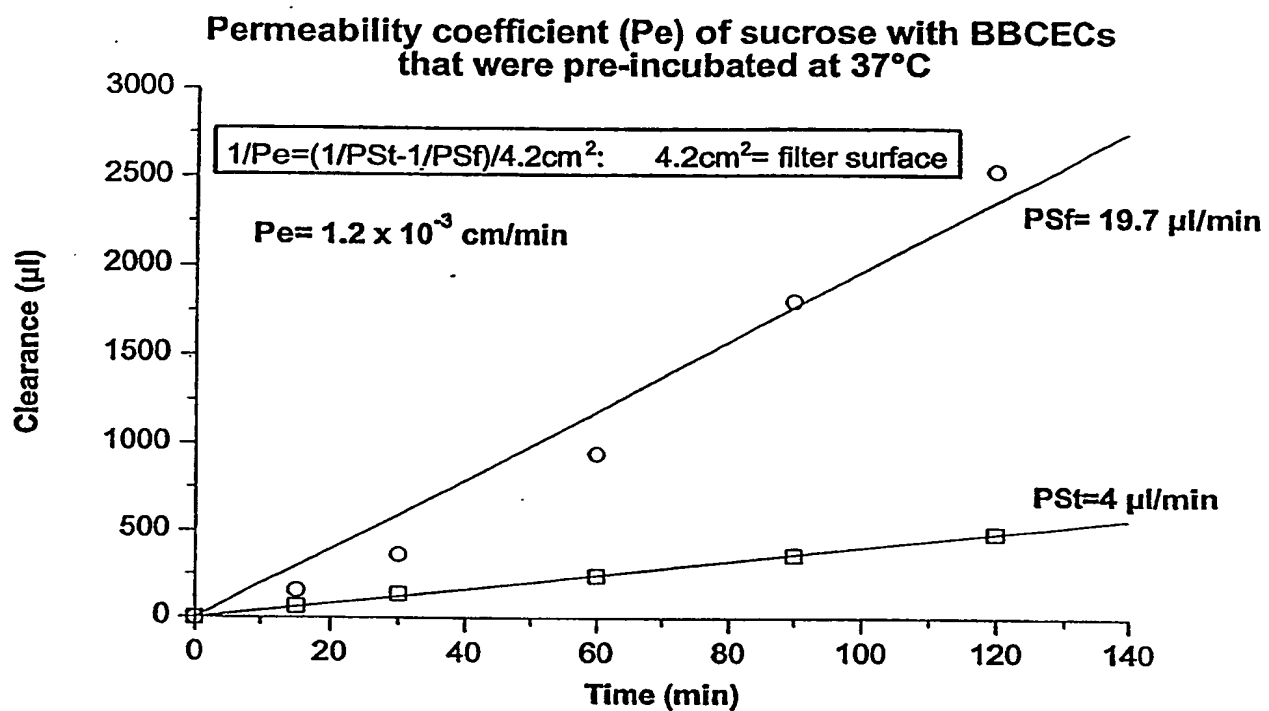


FIGURE 1

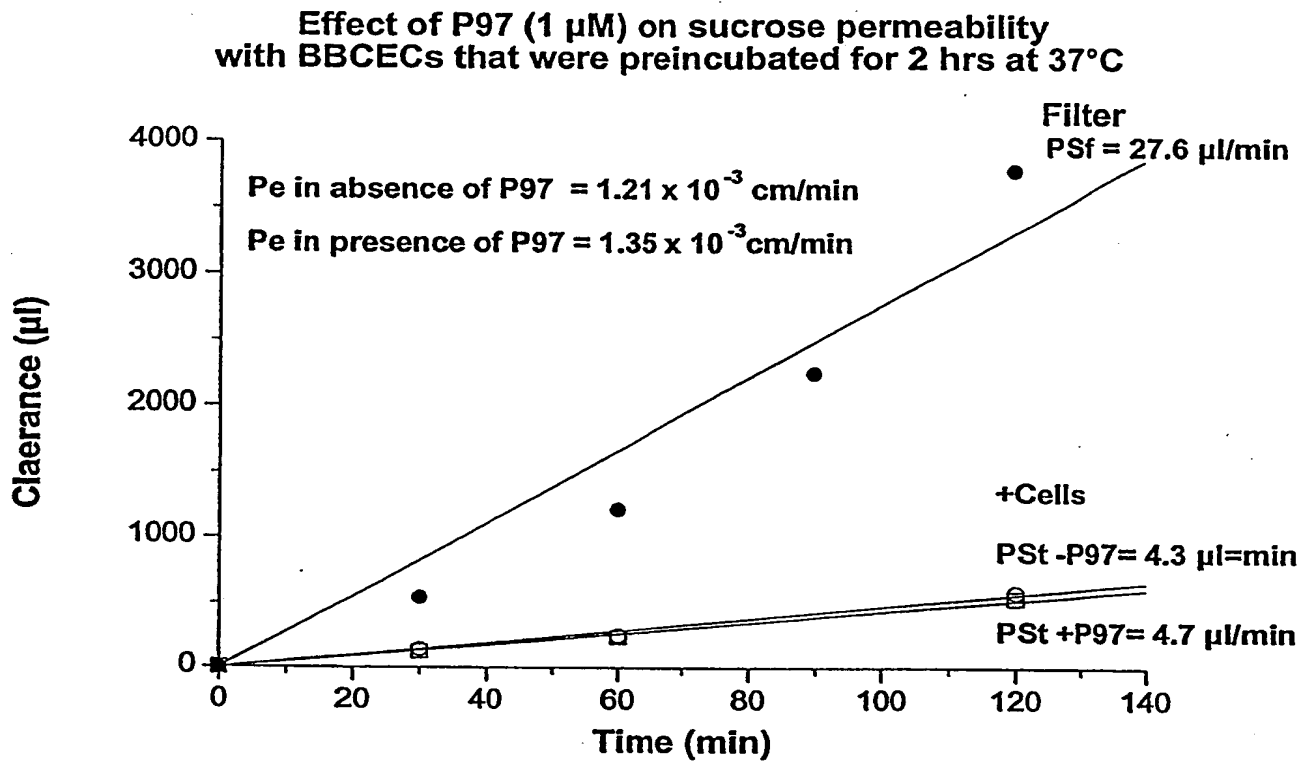


FIGURE 2

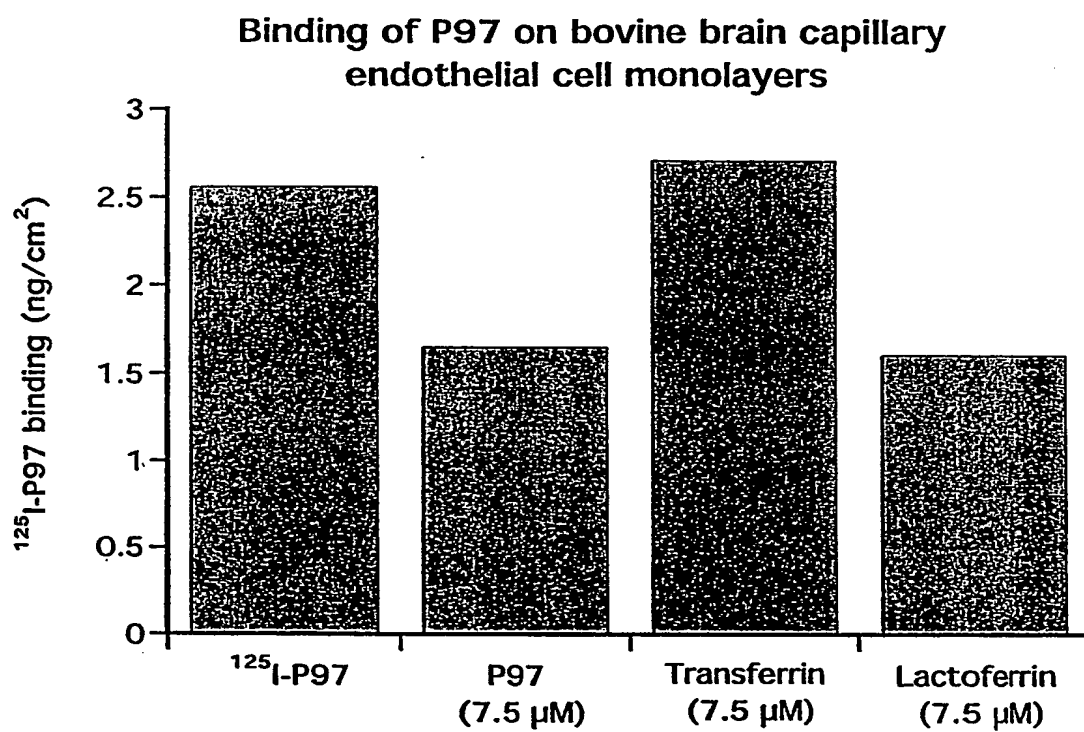


FIGURE 3

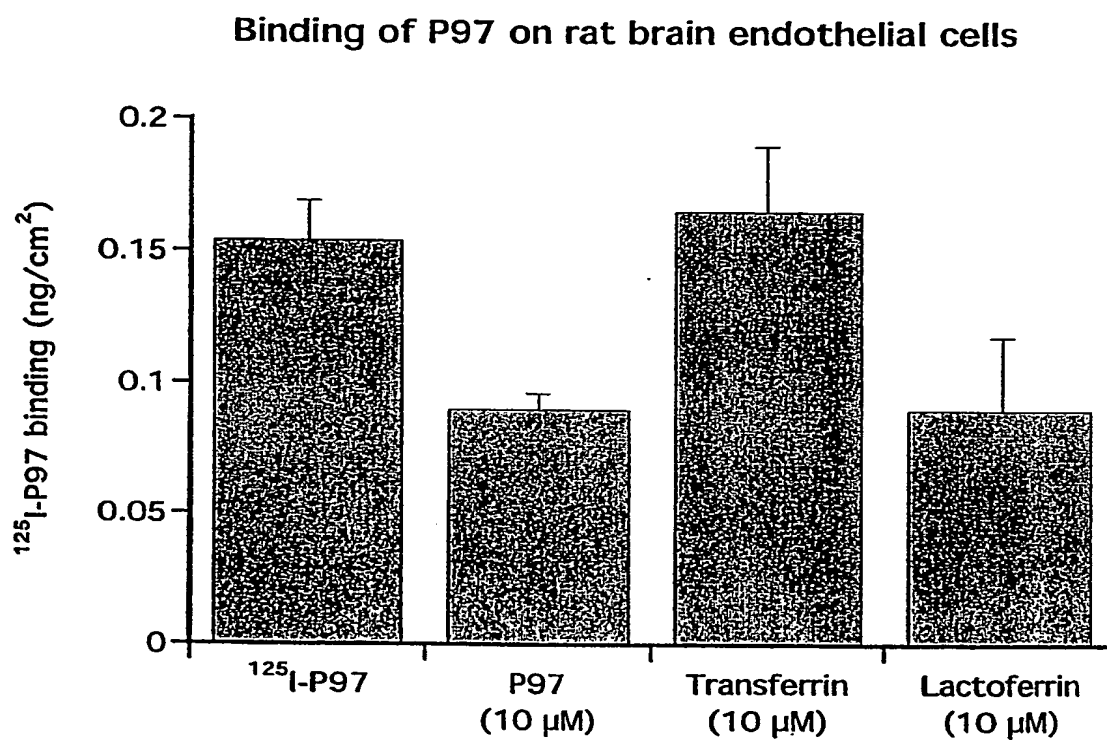


FIGURE 4

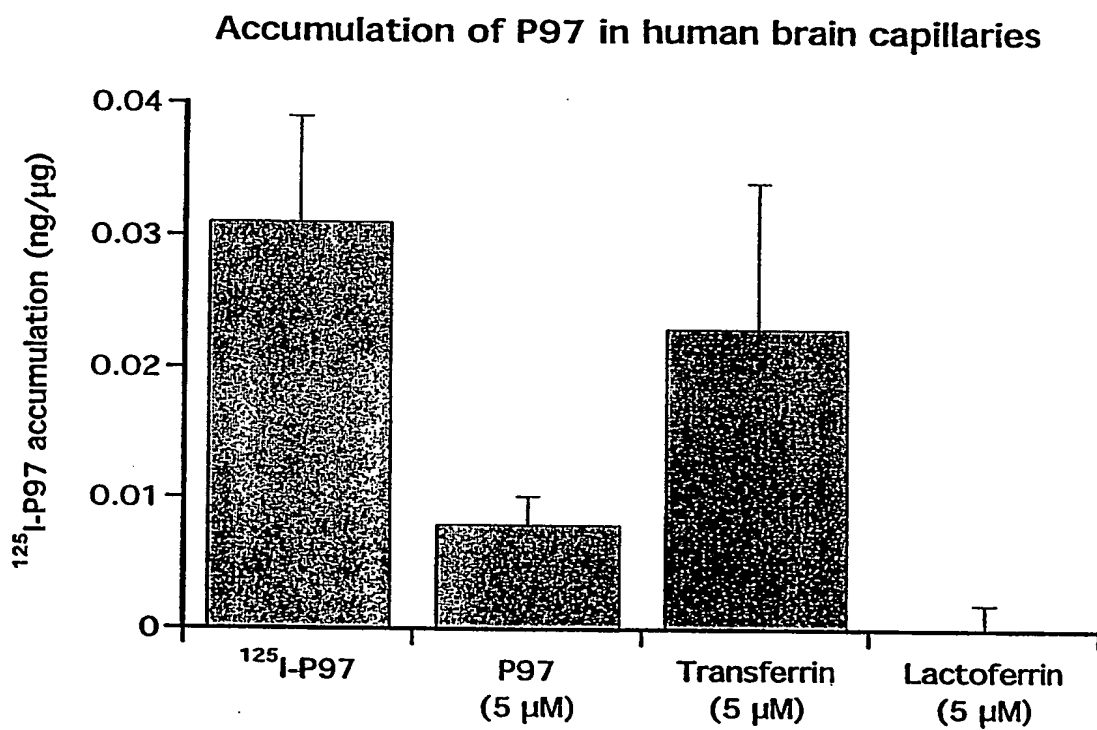


FIGURE 5

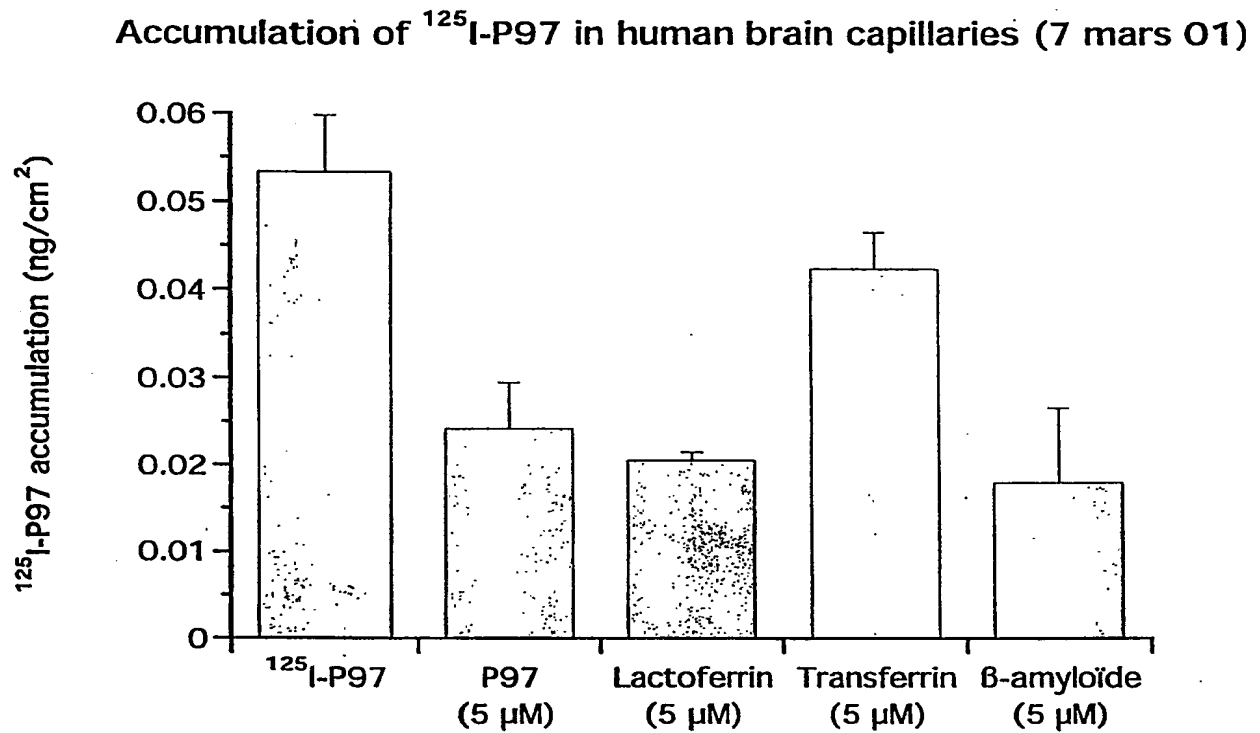


FIGURE 6

Accumulation of P97, lactoferrin and transferrin in BBCE cells

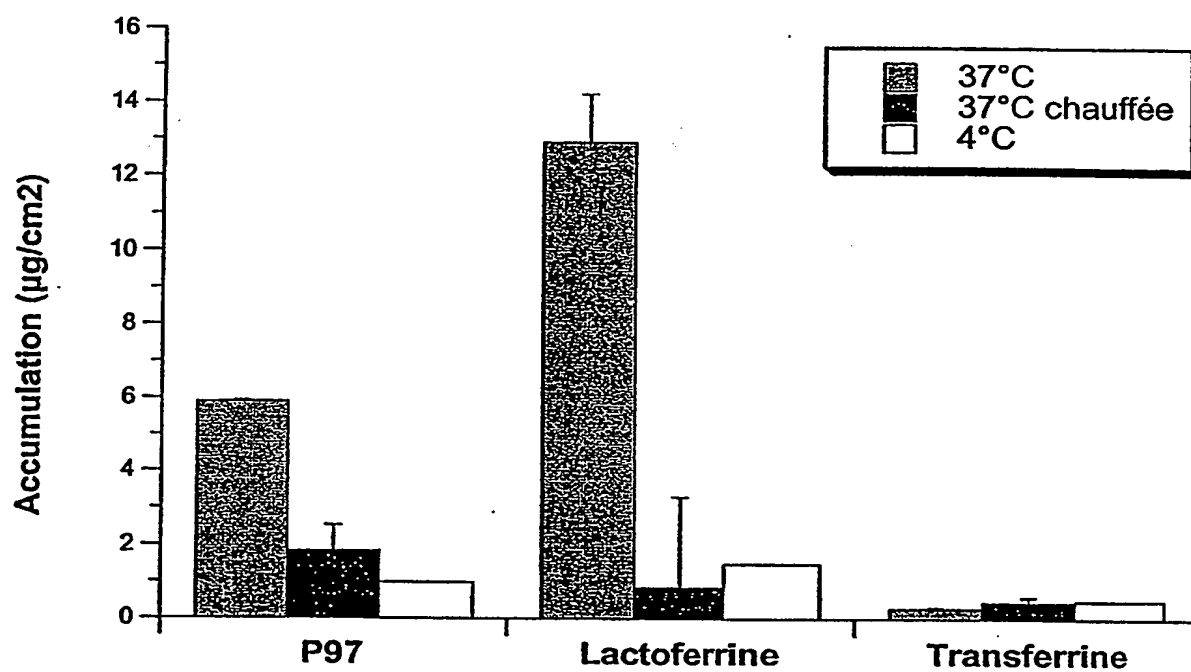


FIGURE 7

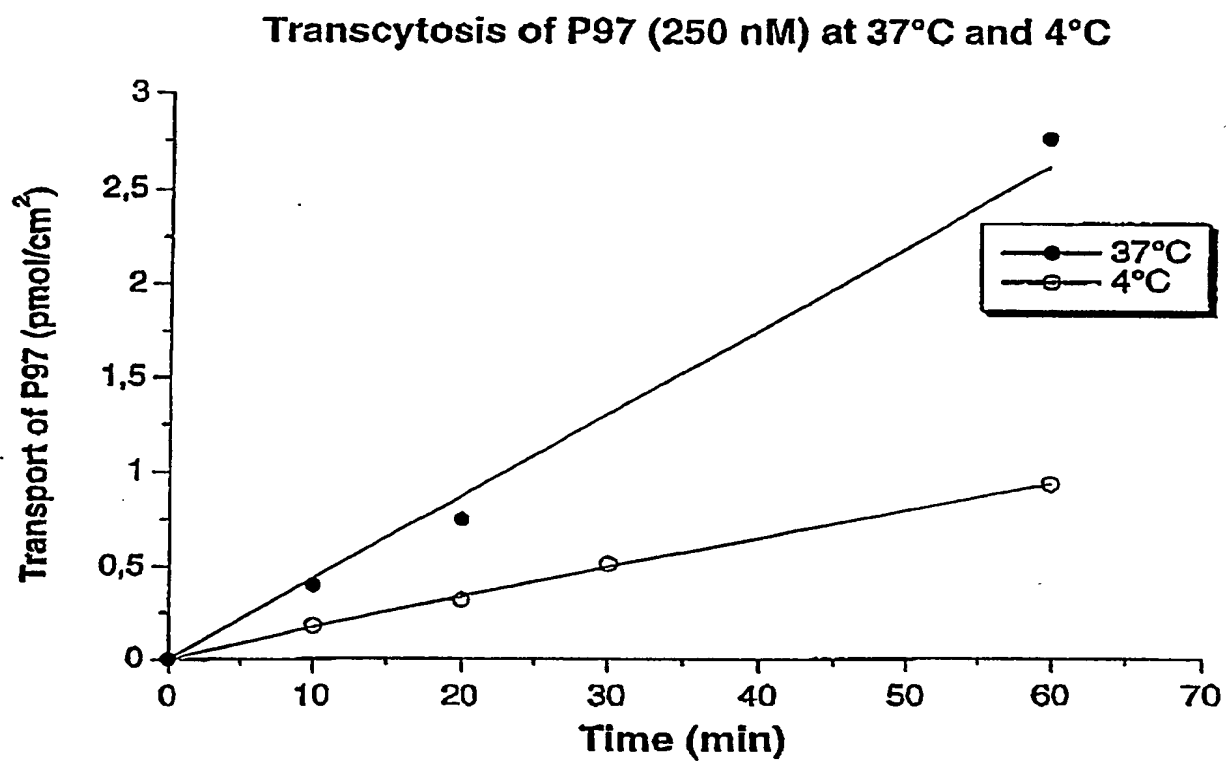


FIGURE 8

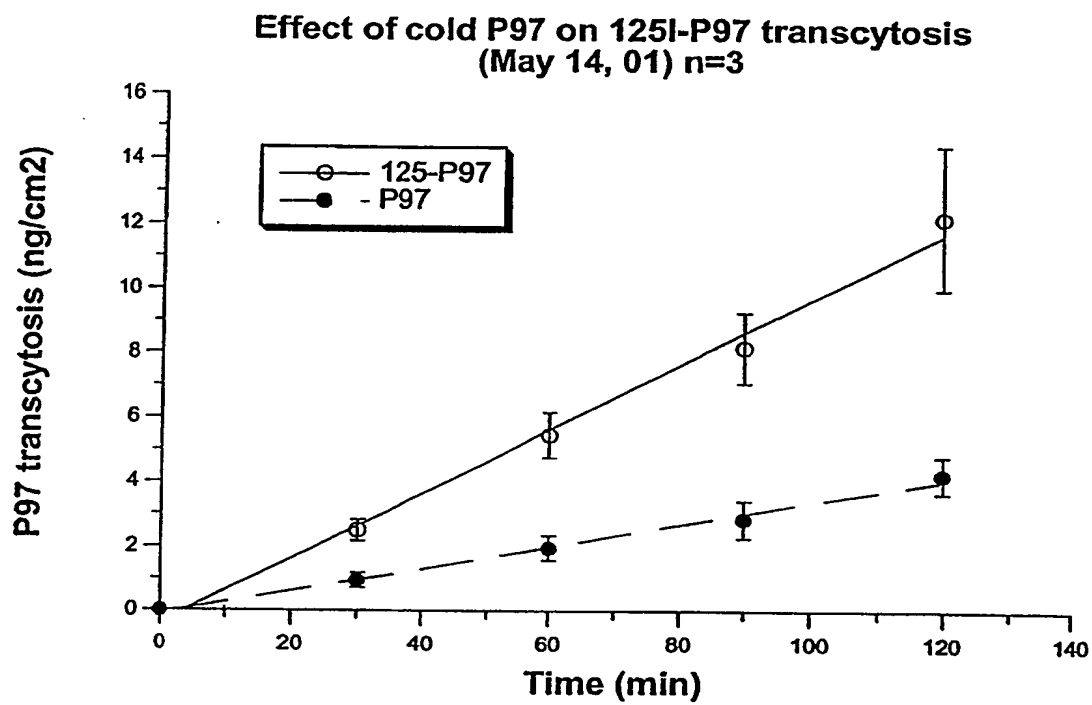


FIGURE 9

Figure 10a

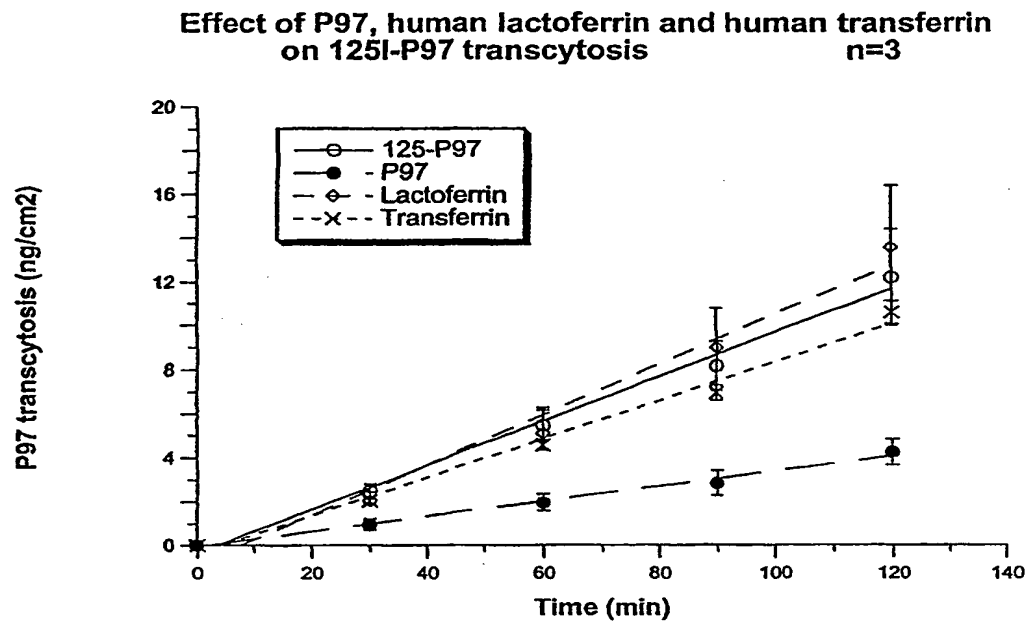


Figure 10 b

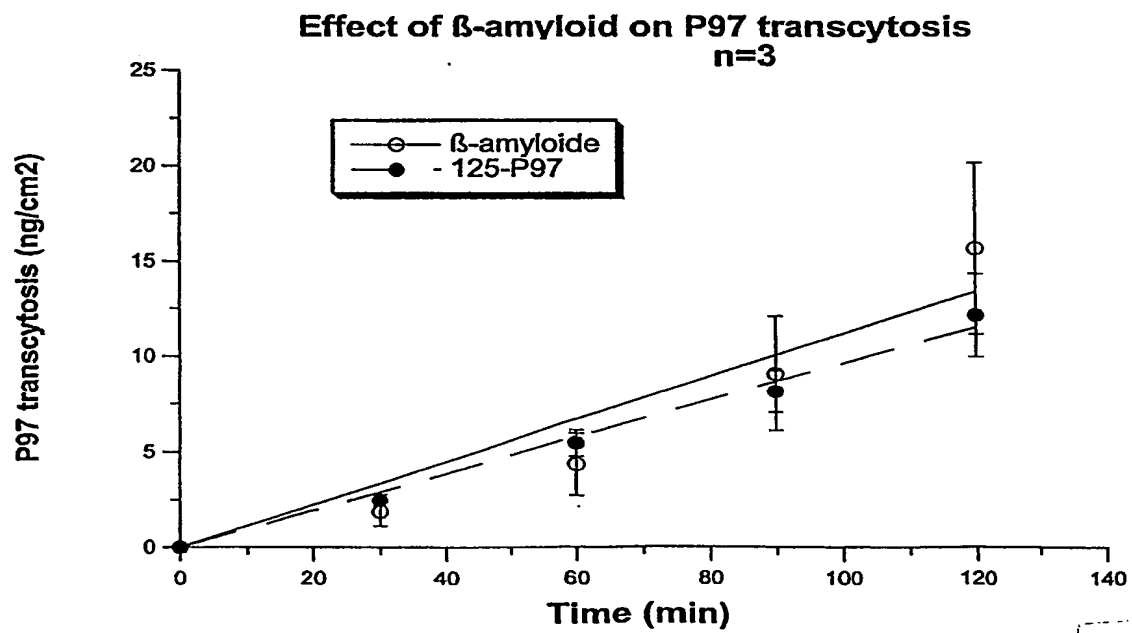
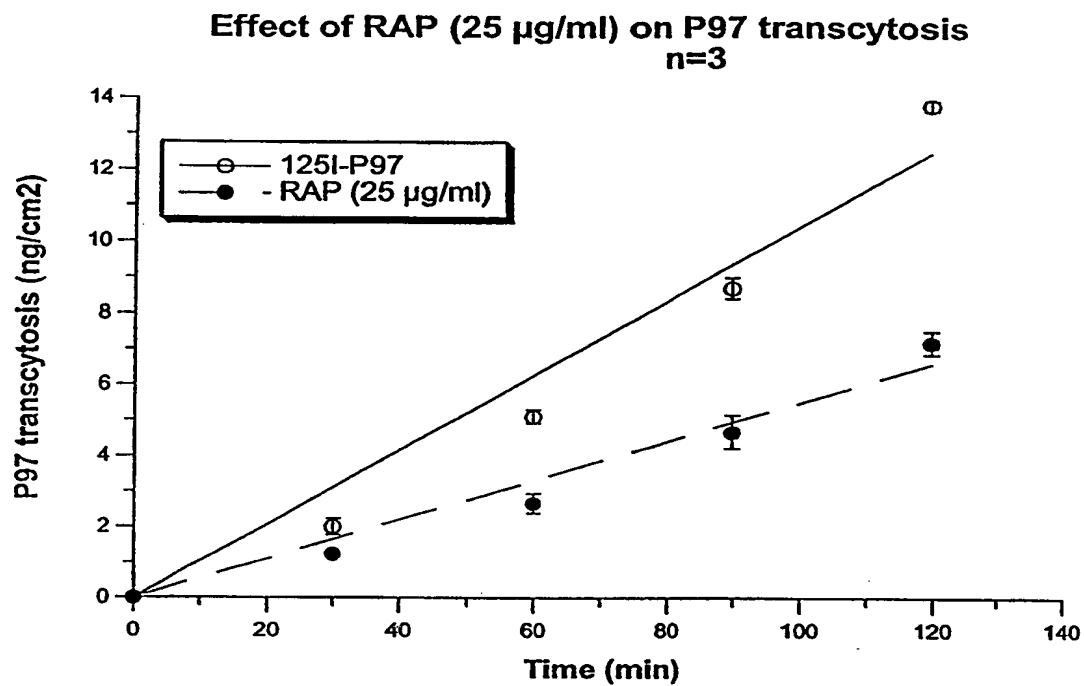


Figure 10c



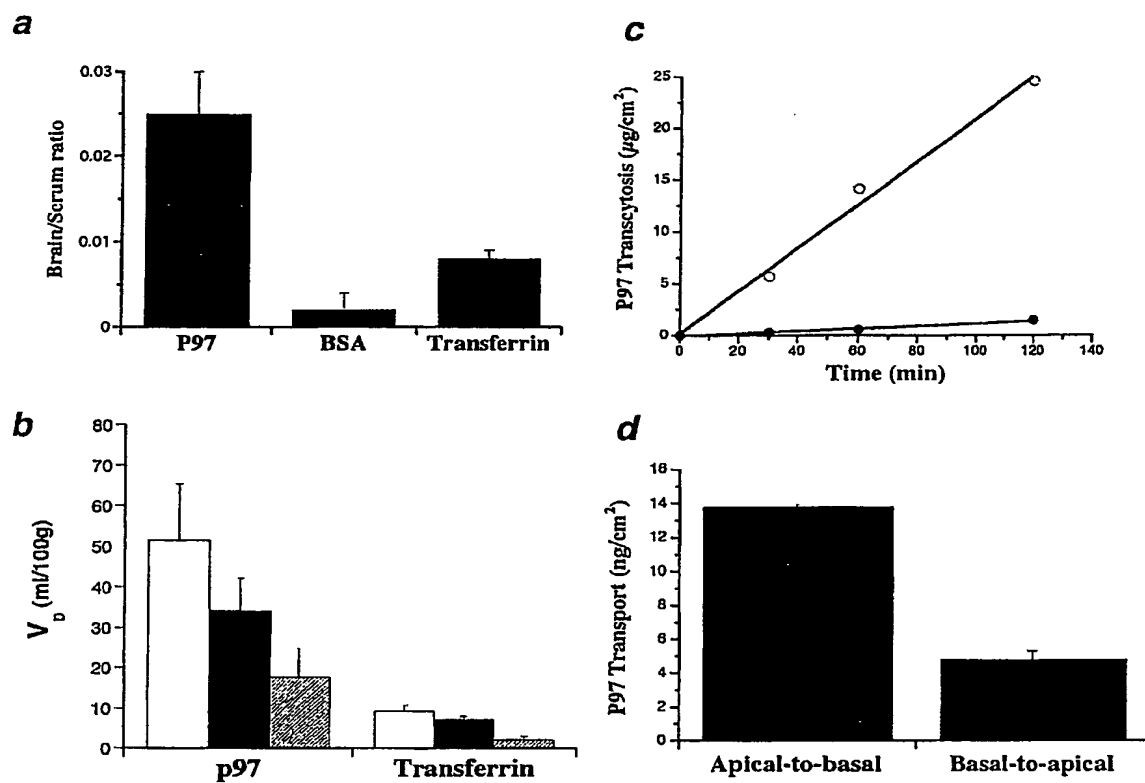


FIGURE 11

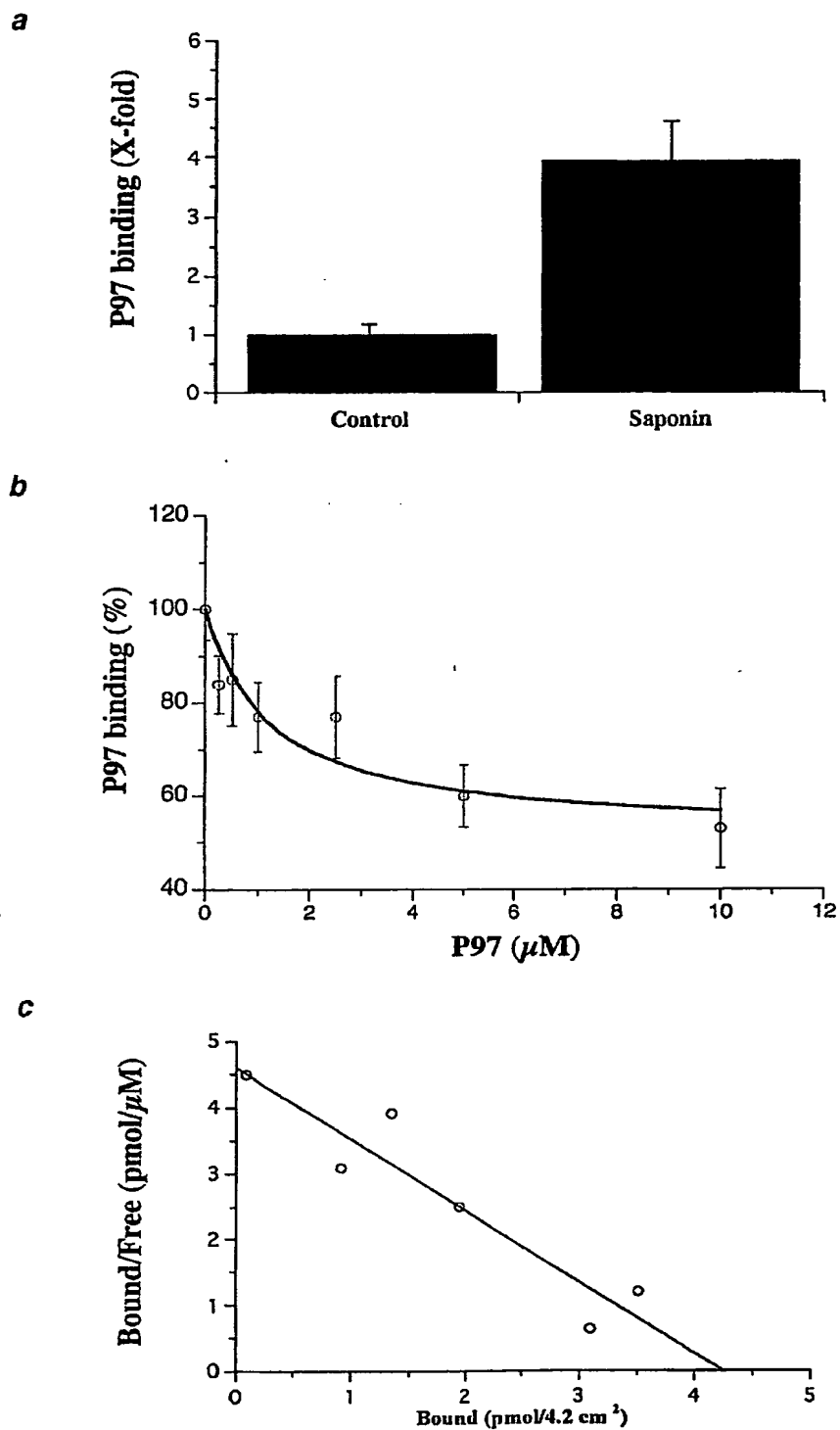


FIGURE 12

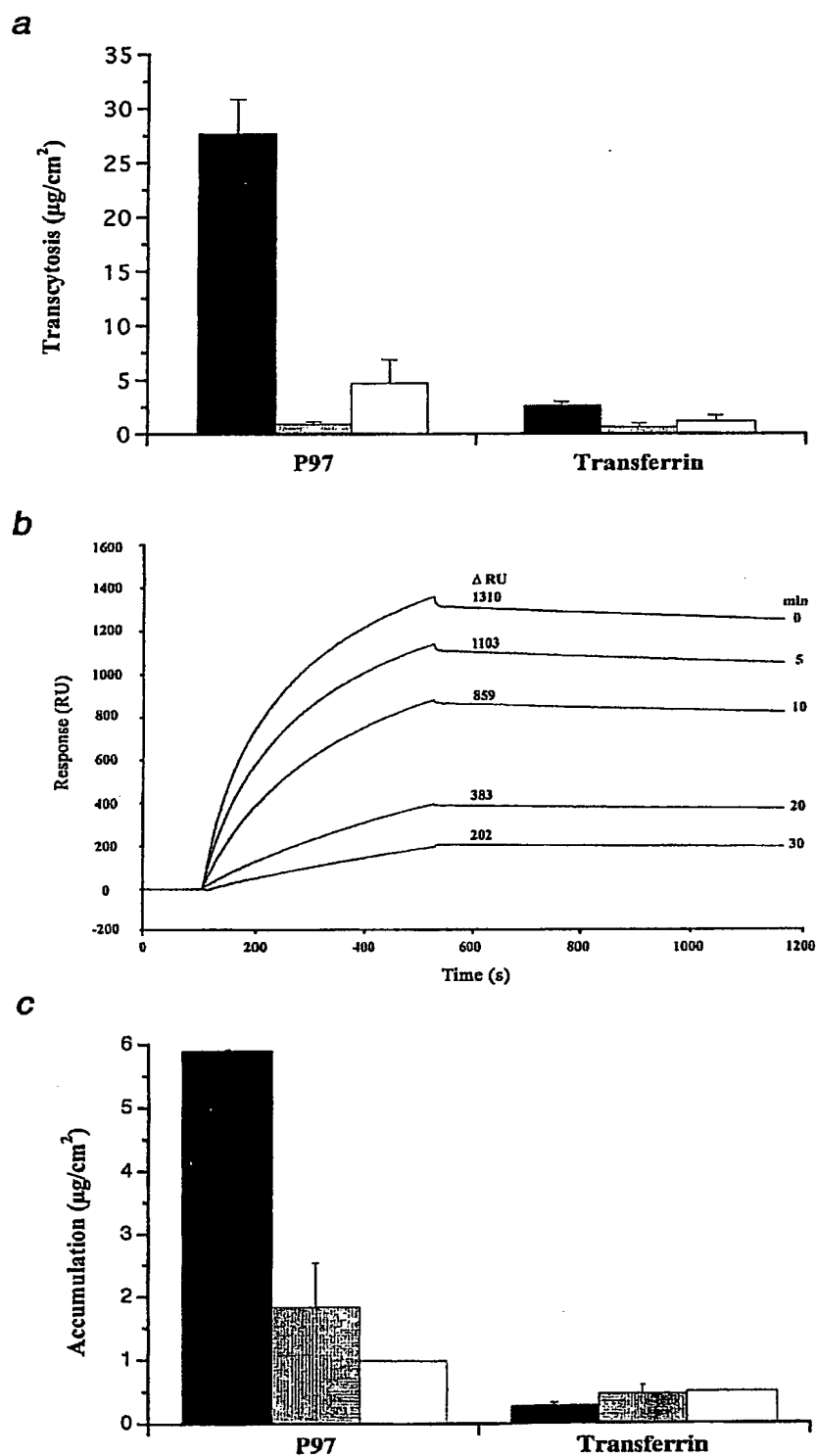


FIGURE 13

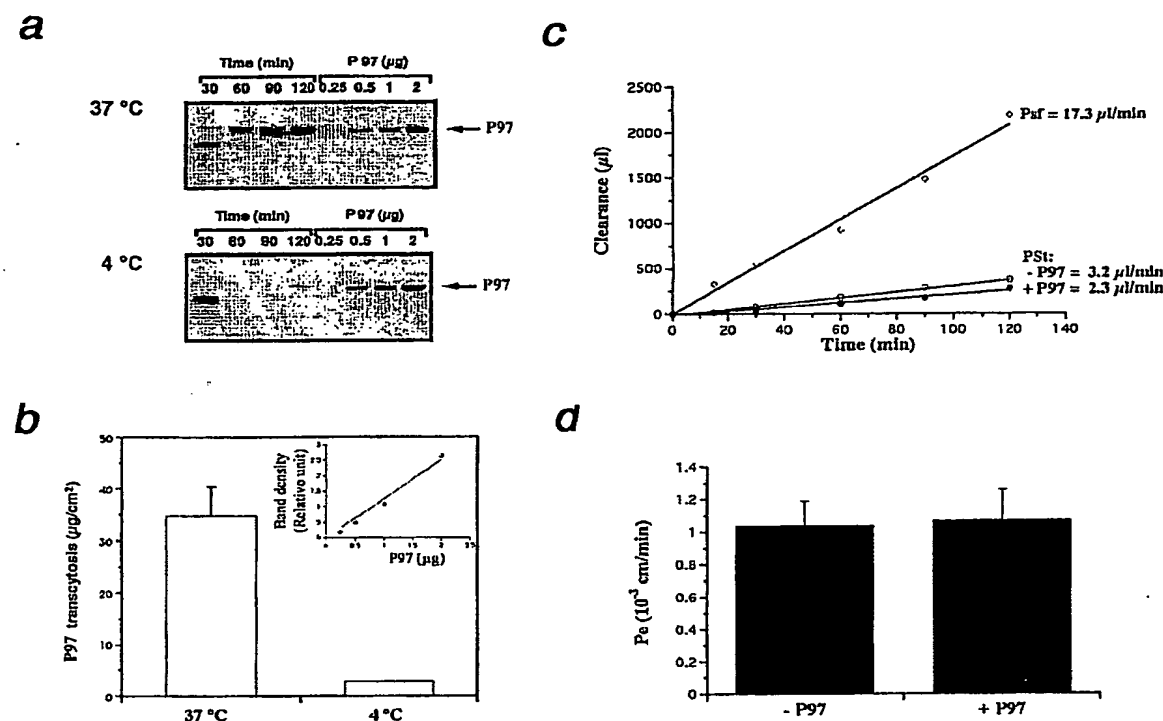


FIGURE 14

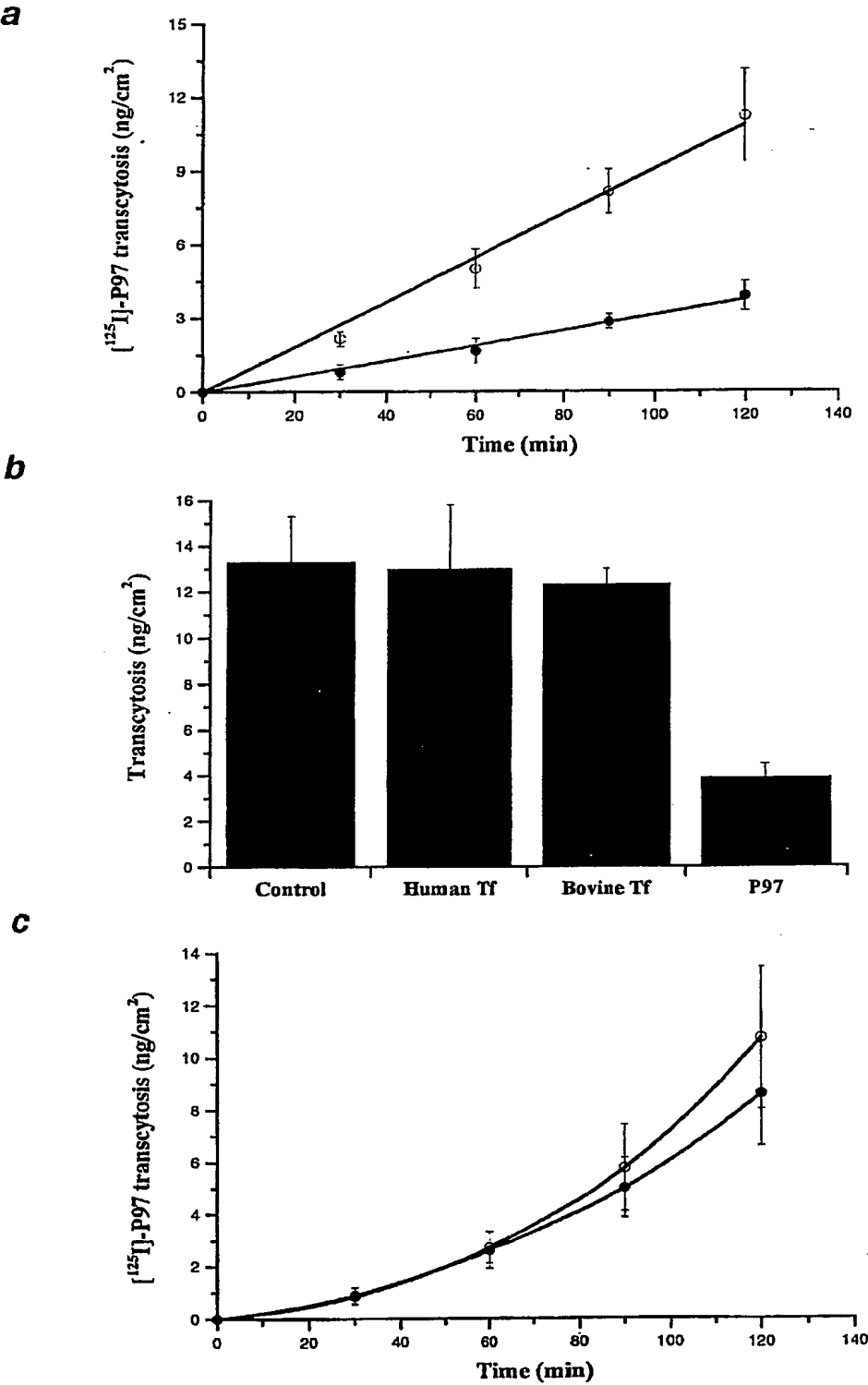


FIGURE 15

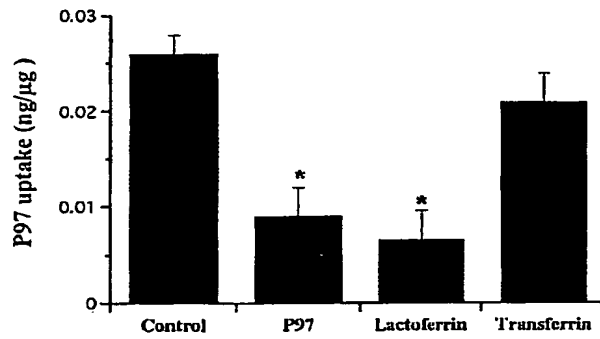
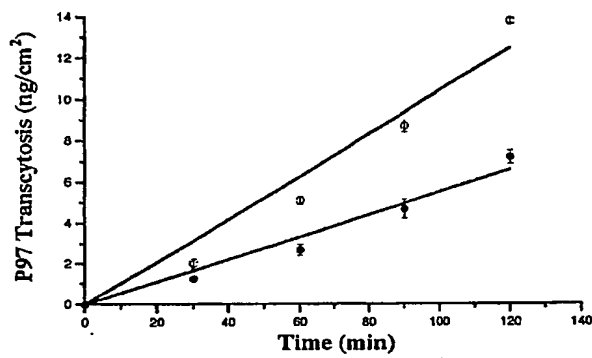
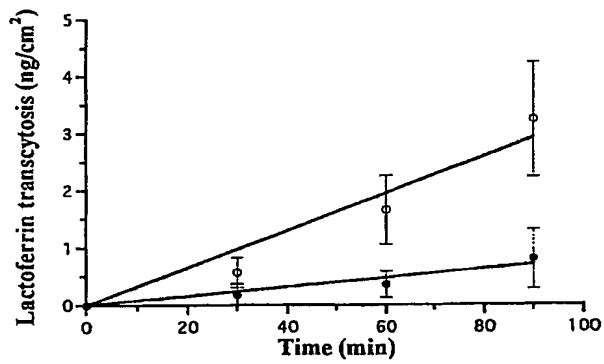
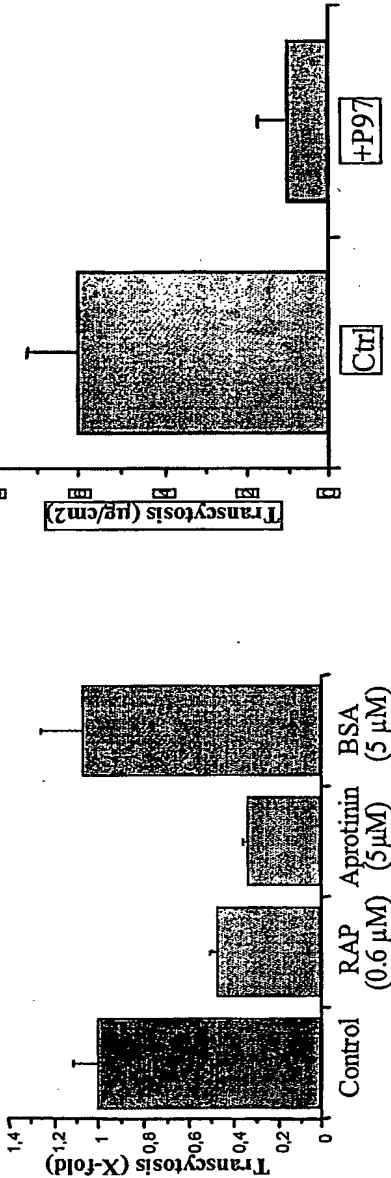
a**b****c**

FIGURE 16

Involvement of LRP on P97 transcytosis

A. Effect of other ligands on P97 transcytosis B. Effect of P97 on lactoferrin transcytosis



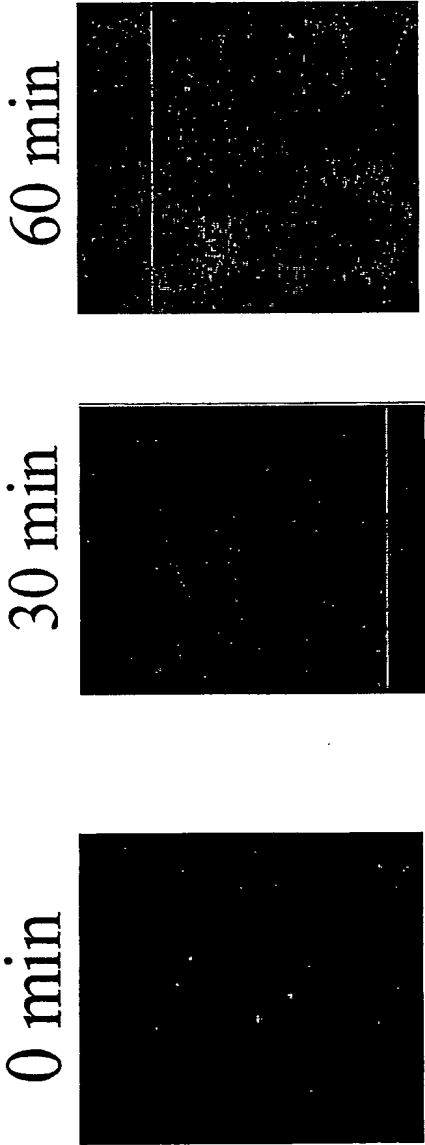
C. Ligands for LRP and megalin

	LRP	Megalin
Lactoferrin	Yes	Yes
RAP	Yes	Yes
Aprotinin	Yes	Yes
BSA	No	Yes

Figure 17

P97 uptake in BBCE

Time course



- Accumulation of P97 in early endosome (perinuclear structure)

FIGURE 18

Estimation of P97 transport rate across the BBB

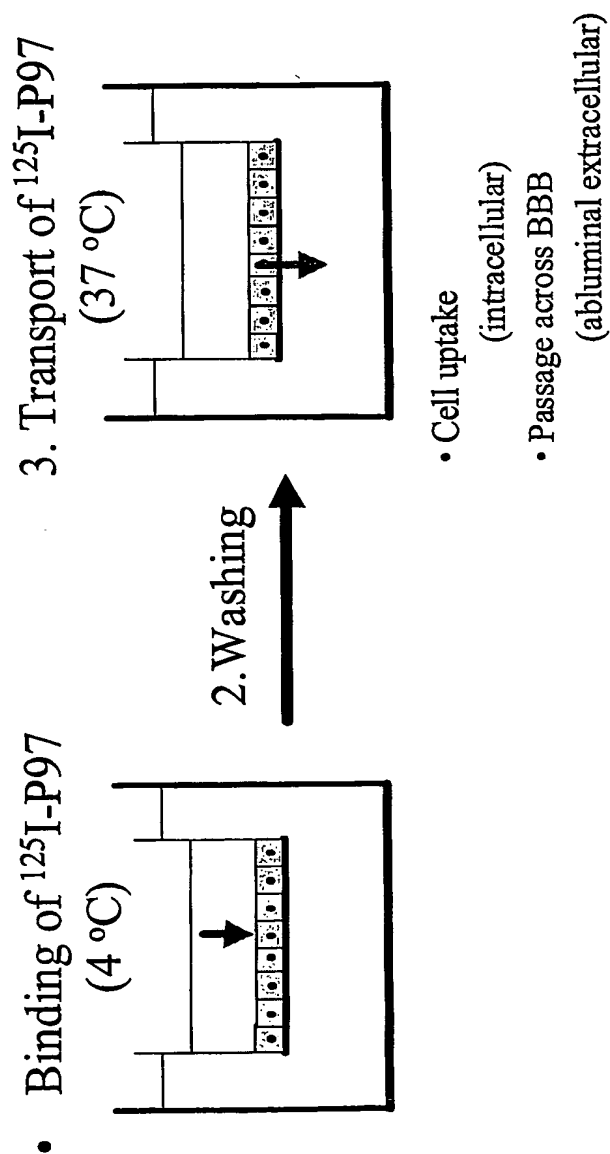
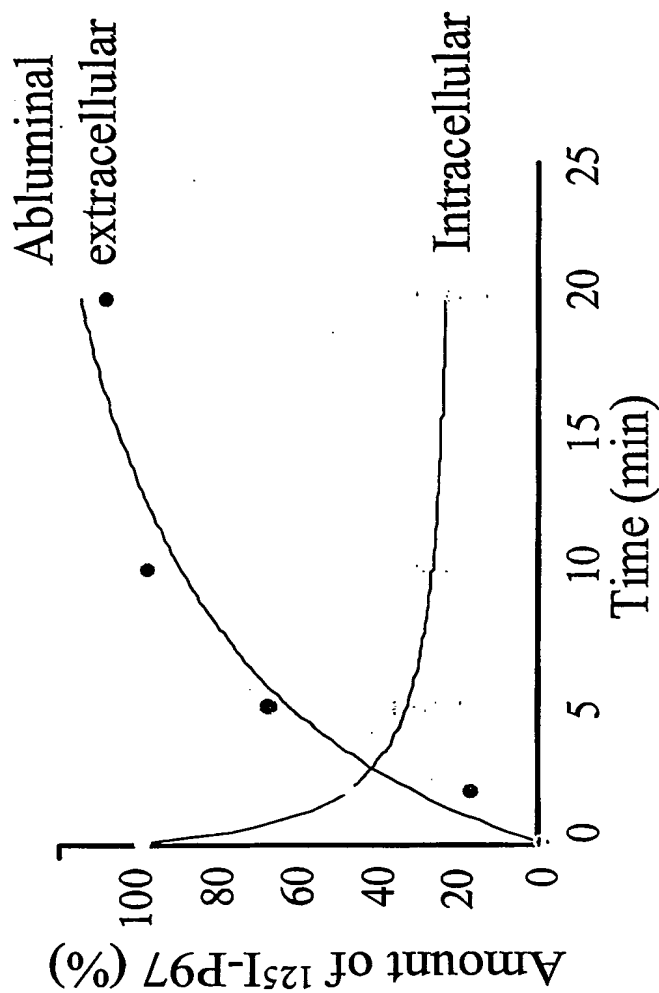


FIGURE 19

Internalization and transcytosis of P97 through BBB model



- Transcytosis of P97 is fast
In 10 min, 80% membrane bound P97 has transcytosed

Figure 20

Transcellular colocalization of P97
and clathrin

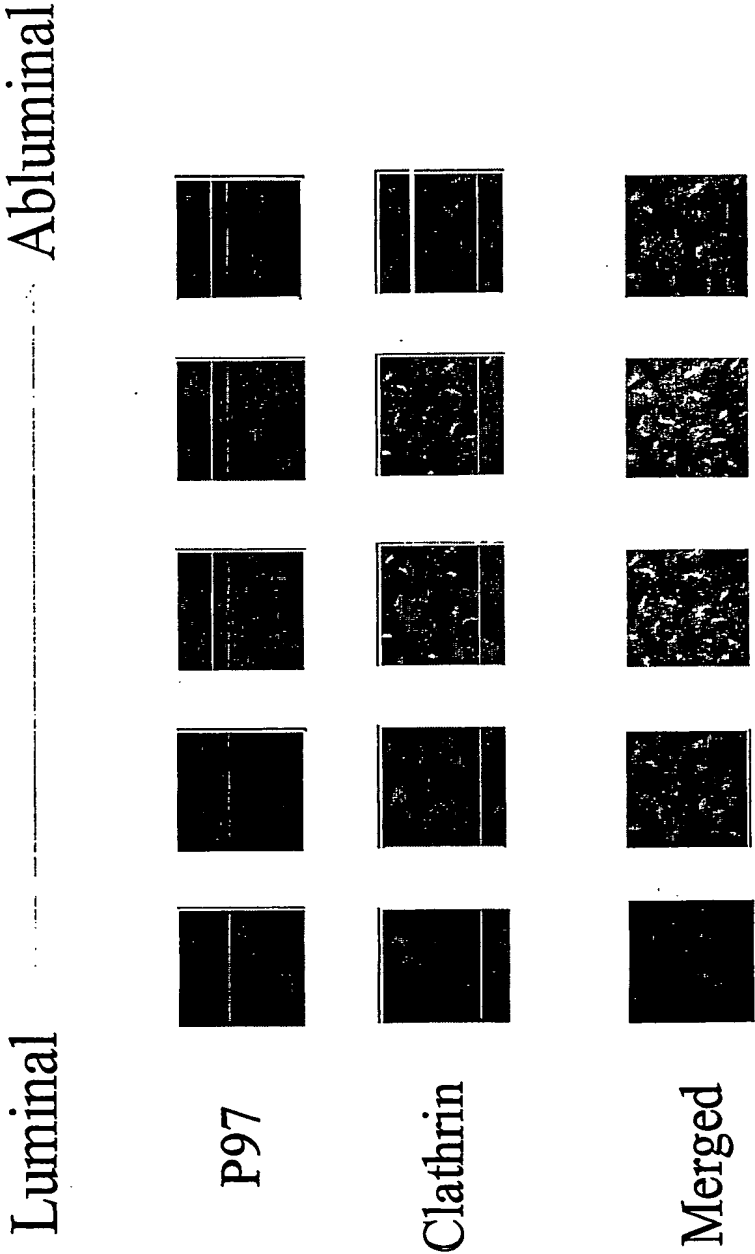


FIGURE 21

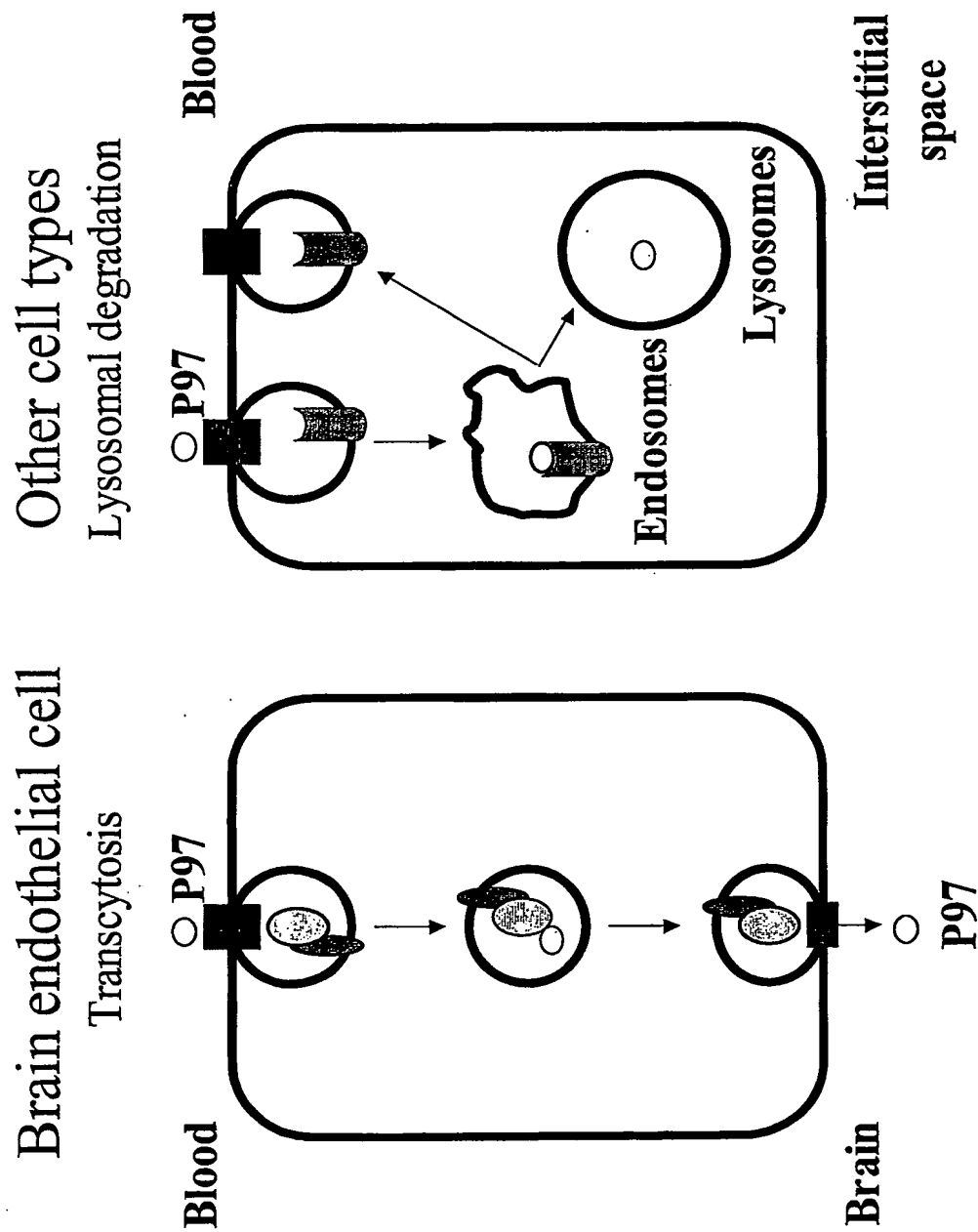


Figure 22

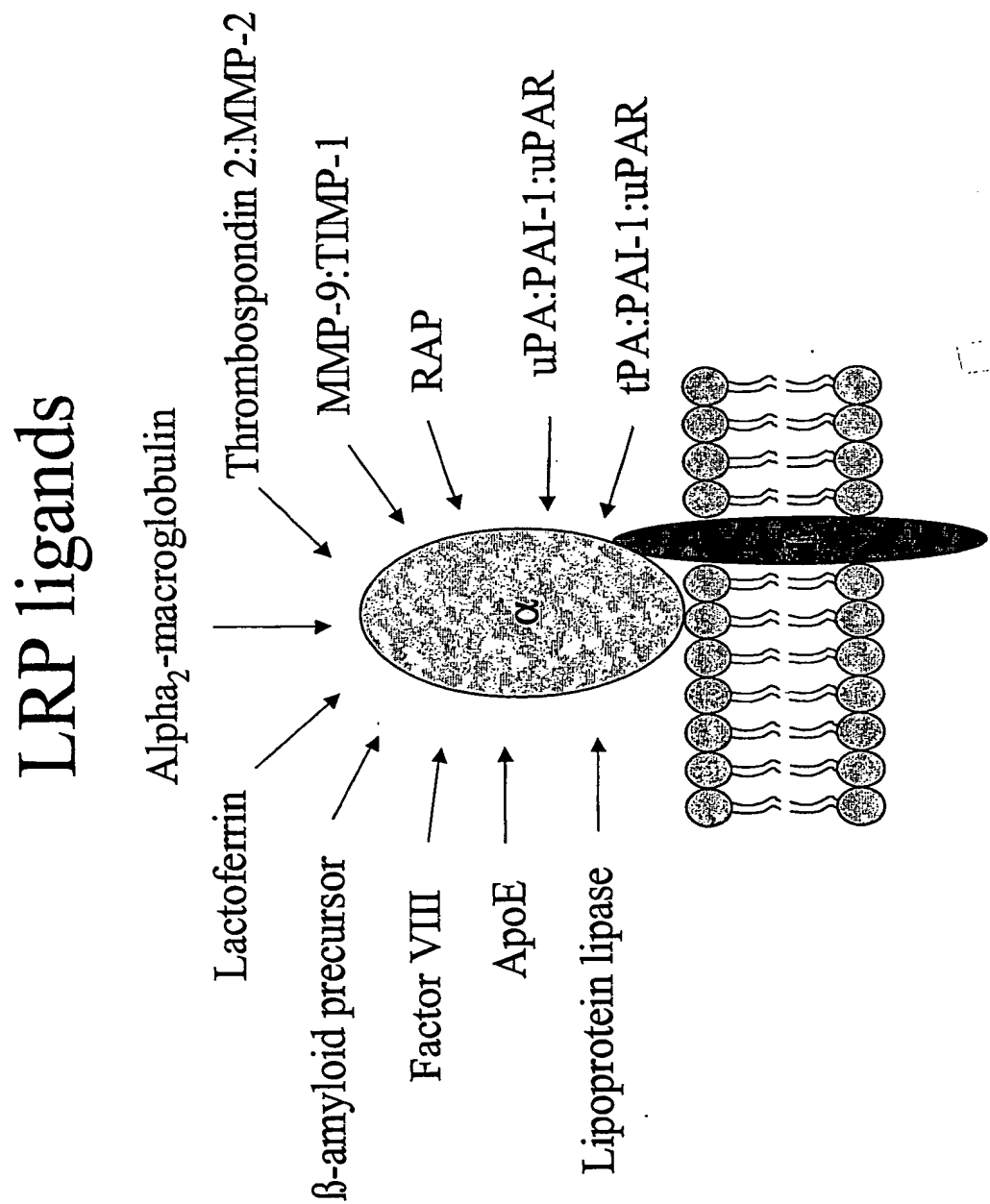


FIGURE 23

Specific uptake of P97 in astrocytes and astrocytomas

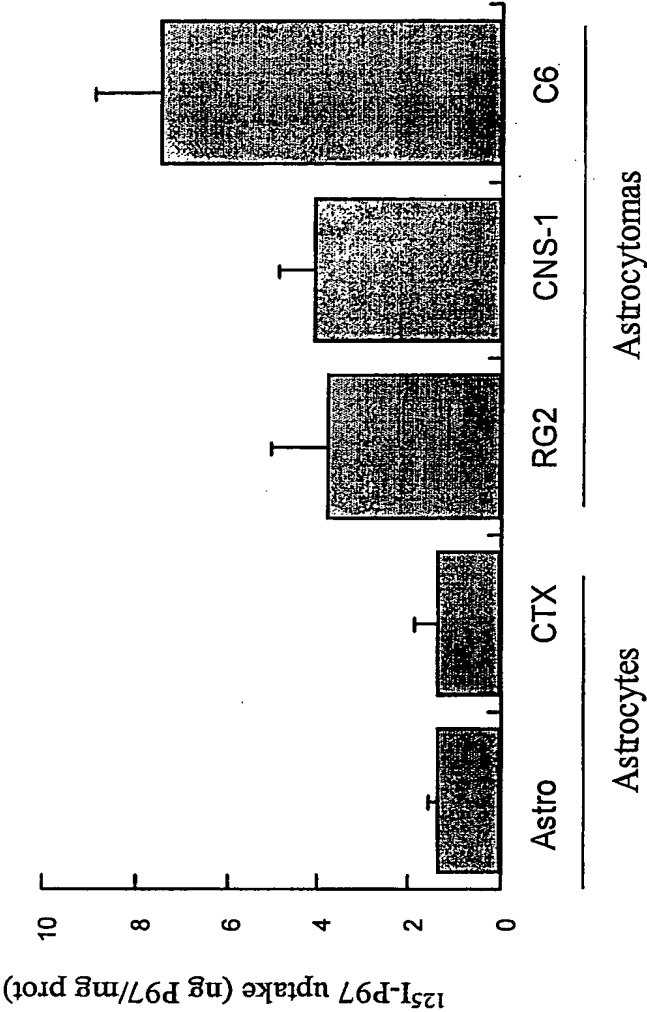
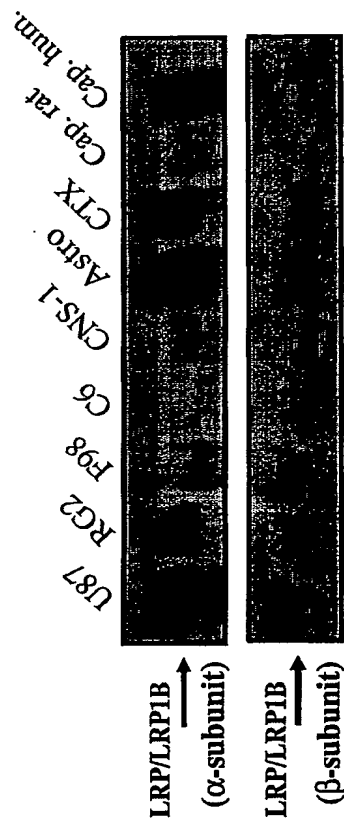


FIGURE 24

Immunodetection of LRP/LRP1B in various astrocytomas, normal astrocytes and brain capillaries

A. Various cell types



B. Rat astrocytes and astrocytomas

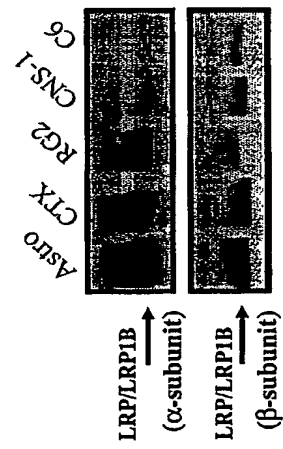
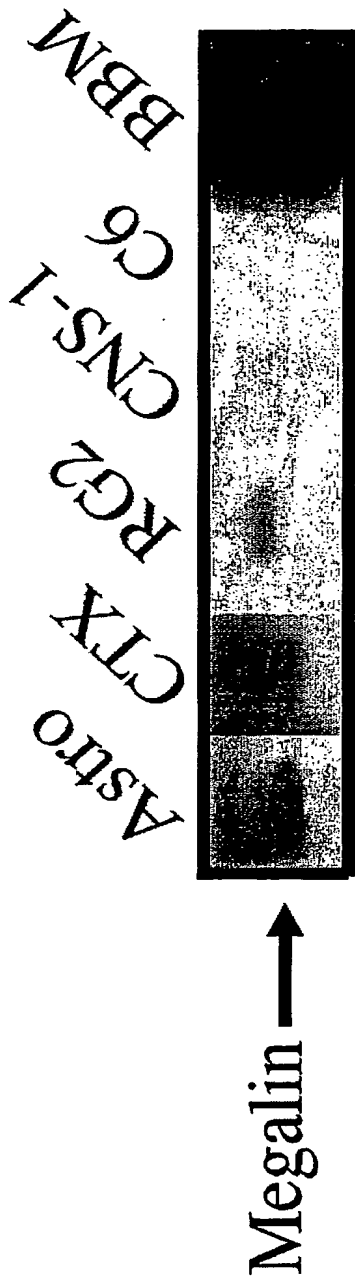


FIGURE 25

Immunodetection of megalin in astrocytes and astrocytomas



• BBM: renal brush-border membranes (positive control)

FIGURE 26

LRP1B expression in astrocytes and astrocytomas (RT-PCR)



FIGURE 27

Correlation between P97 uptake and LRP1B expression

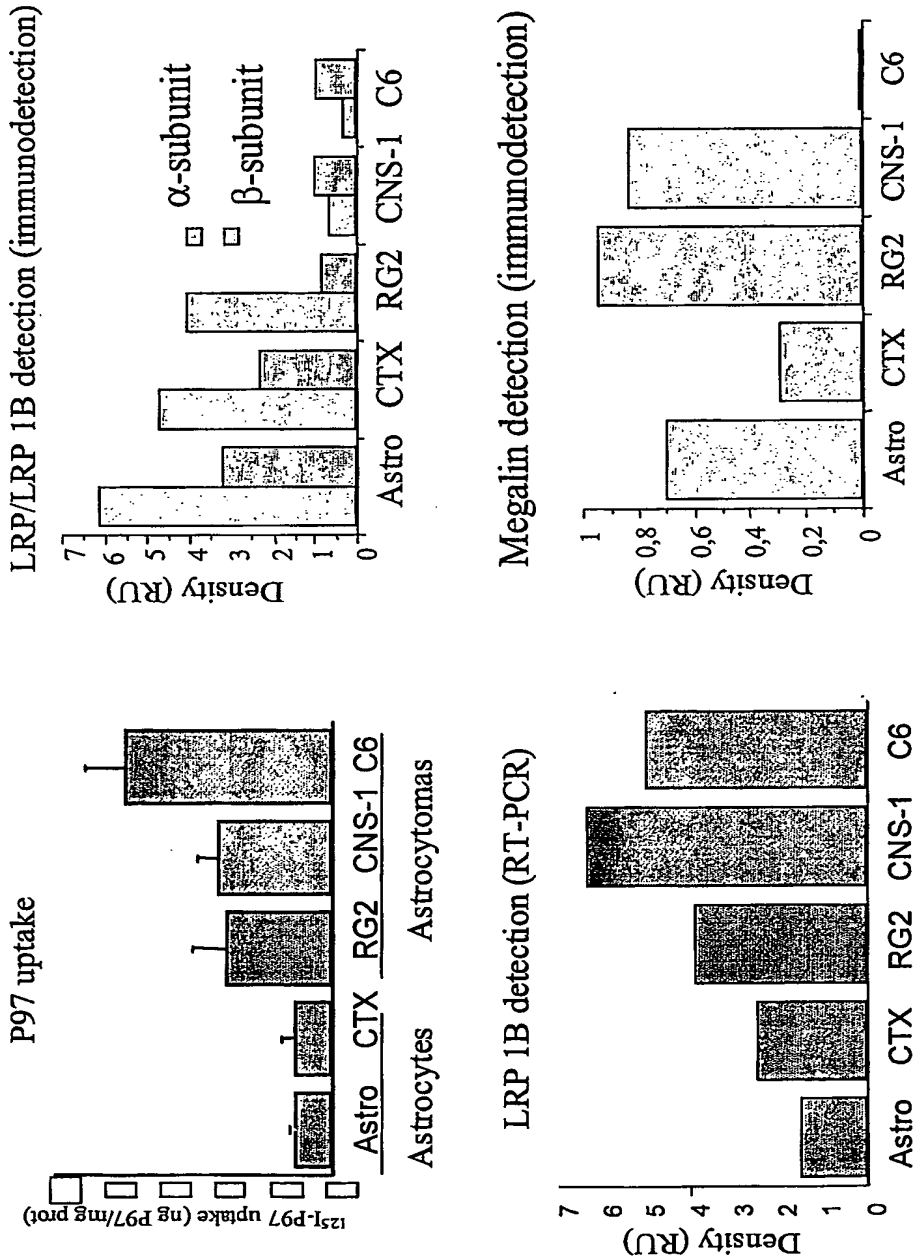


FIGURE 28

LRP/LRP1B migrates as a high molecular weight dissociable complex in presence of P97

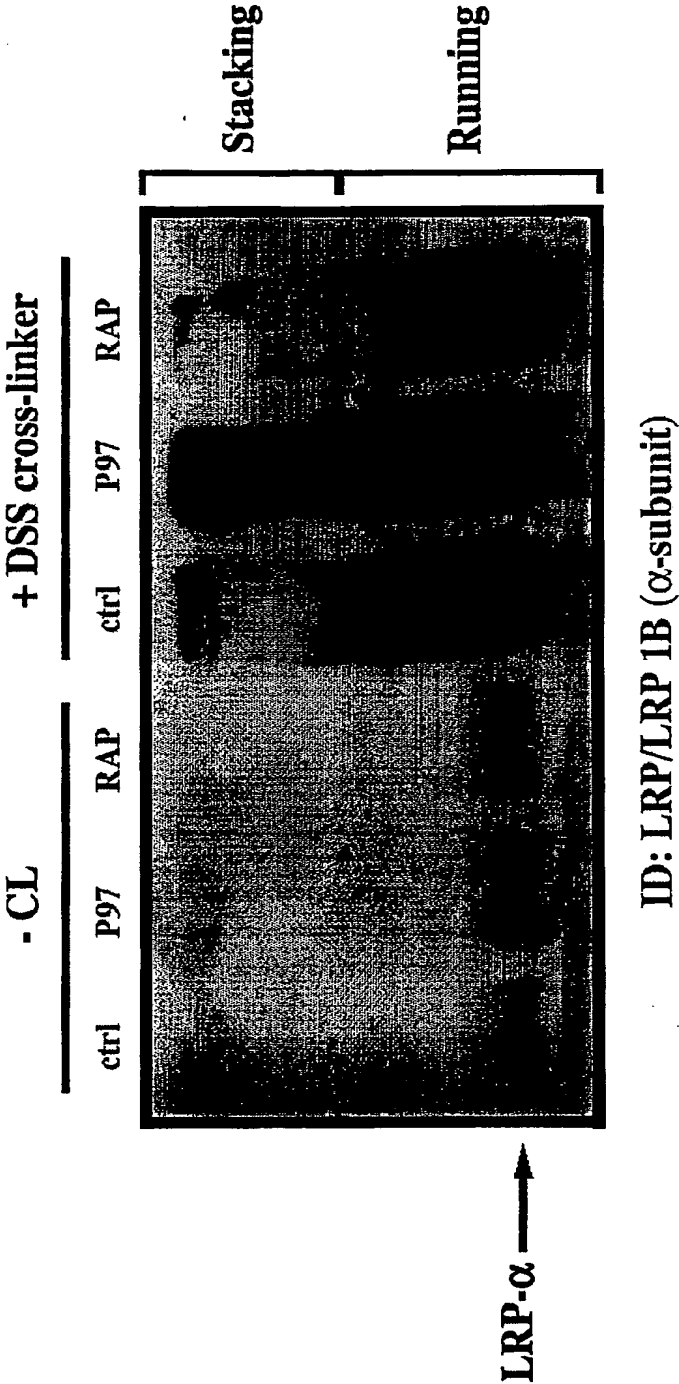


FIGURE 29

Reducing condition induces release of P97
from high molecular weight complex

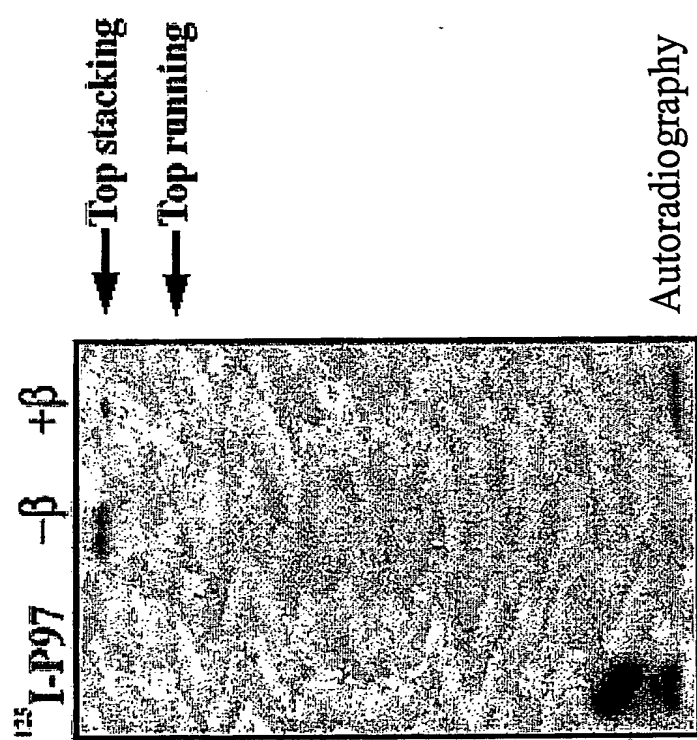


FIGURE 30

P97 migrates as a high molecular weight protein complex

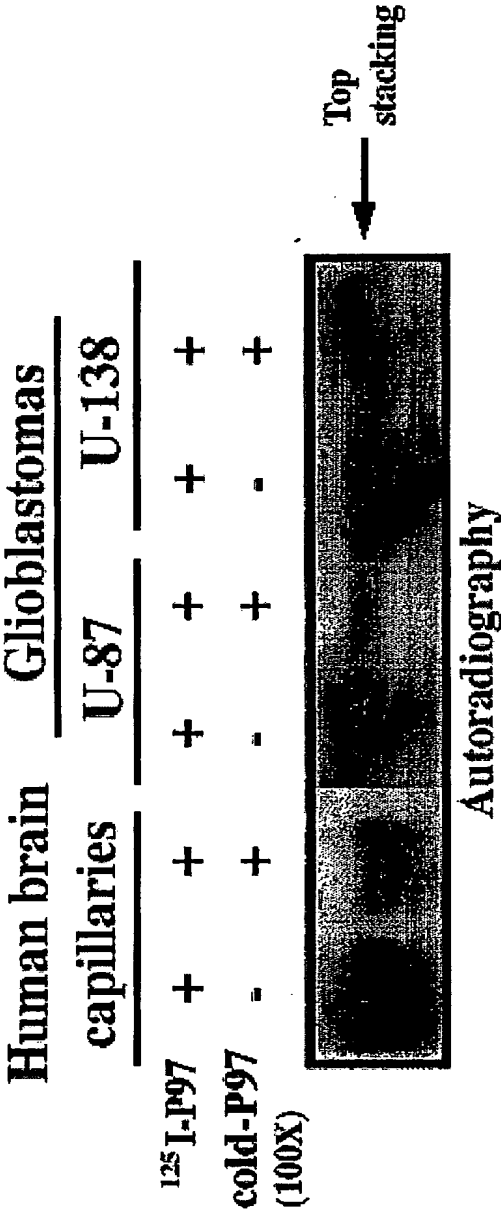


FIGURE 31

Expression of members of LDL receptor family
(RT-PCR)

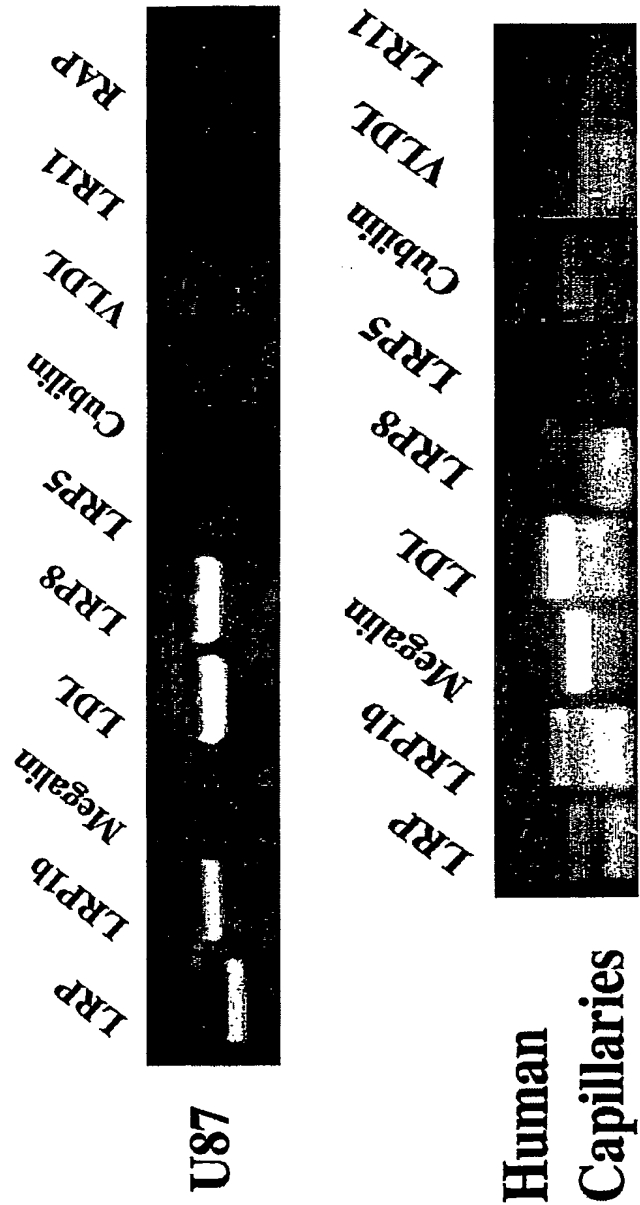
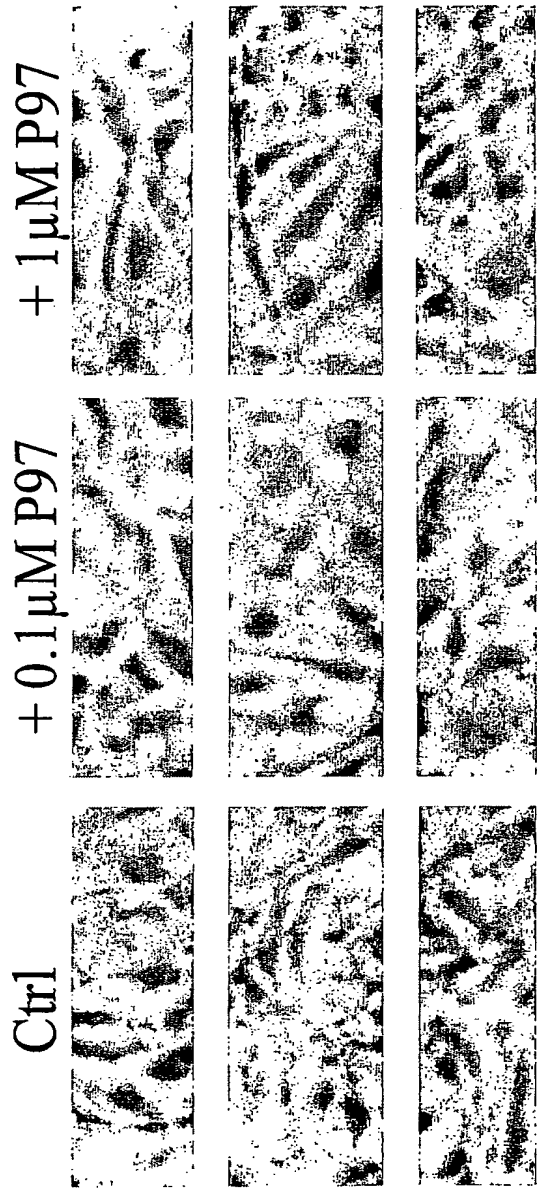


FIGURE 32

No morphological changes after 72 hrs
treatment with P97 in U-87 cells



- 1 μ M P97 = 80 μ g/mL
- Plasmatic concentration:
 - P97 \approx 3-15 ng/mL
 - Transferrin \approx 3 mg/mL
 - Lactoferrin \approx 100-300 ng/mL

FIGURE 33

Effect of P97 and RAP treatments
in U-87 cells

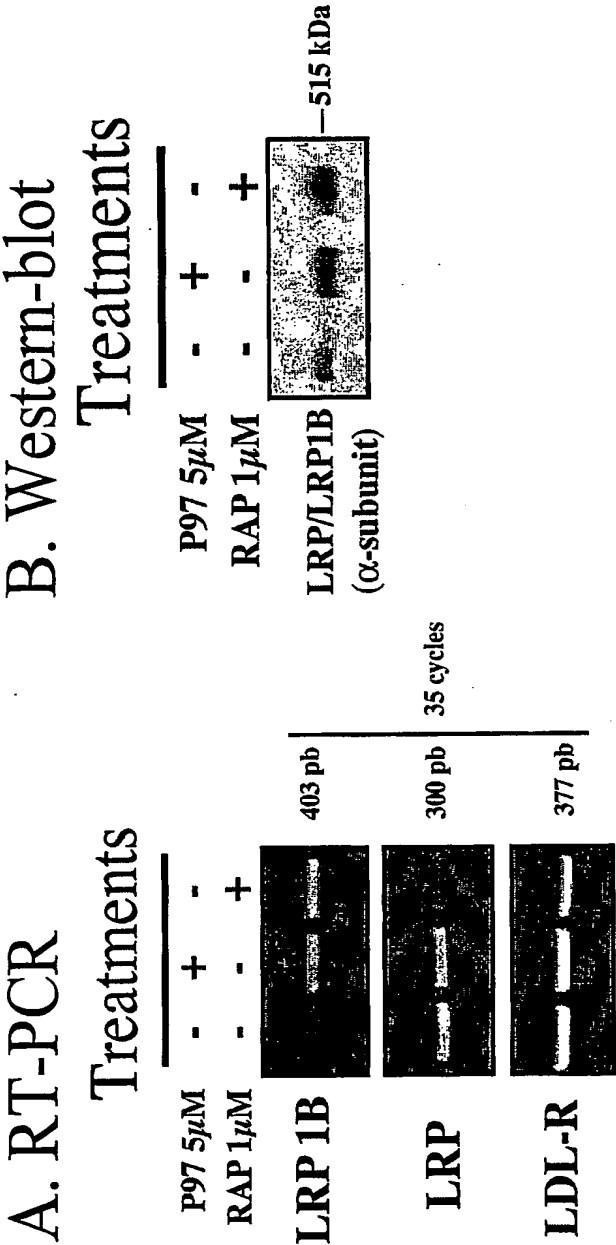


FIGURE 34

Effect of P97 and RAP treatment in U-87 cells
(RT-PCR)

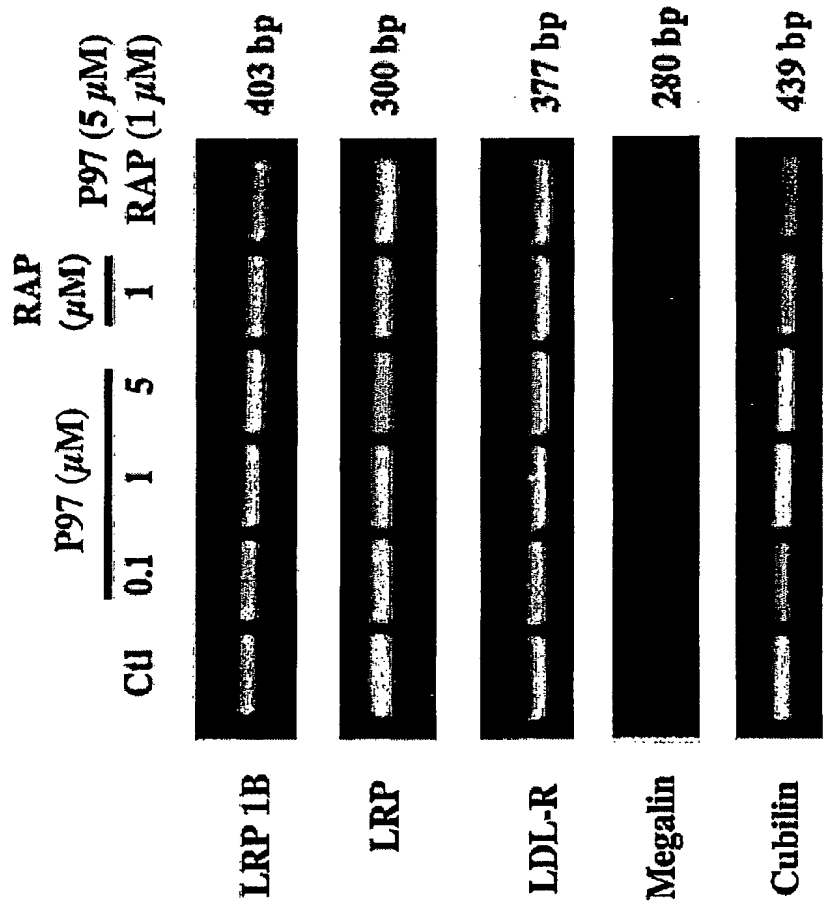


FIGURE 35

Effect of P97 and RAP treatment in U-87 cells (RT-PCR)

Effect of P97 and RAP treatment
in U-87 cells

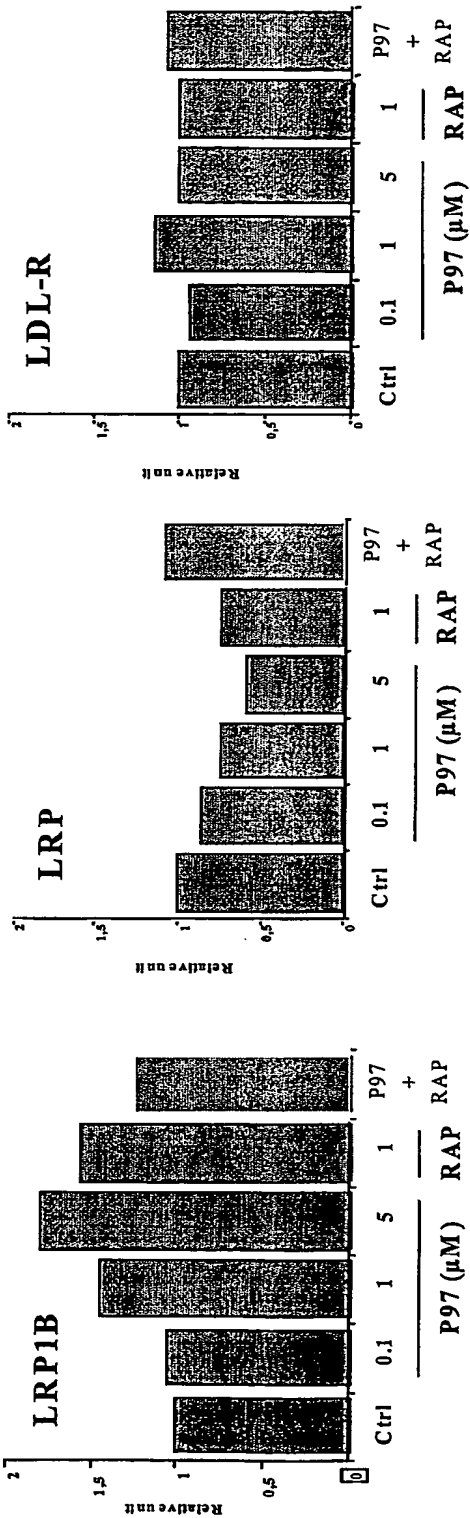


FIGURE 37

Effect of P97 and RAP treatment in U-87 cells

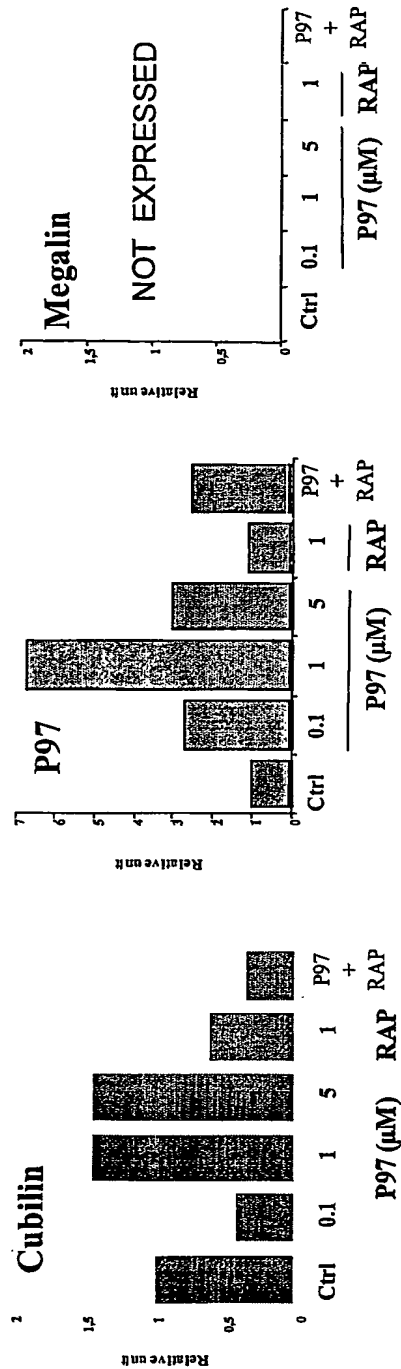


FIGURE 38

Expression of members of
the LDL receptor family in
MG1391



FIGURE 39

Effect of P97 and RAP treatment in MG-1391 cells
(RT-PCR)

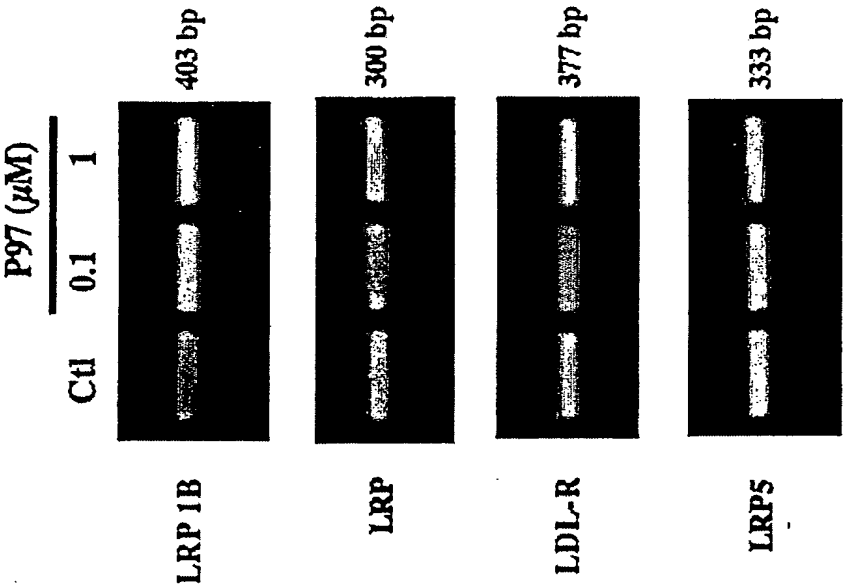


FIGURE 40

Effect of P97 and RAP treatment in MG-1391 cells

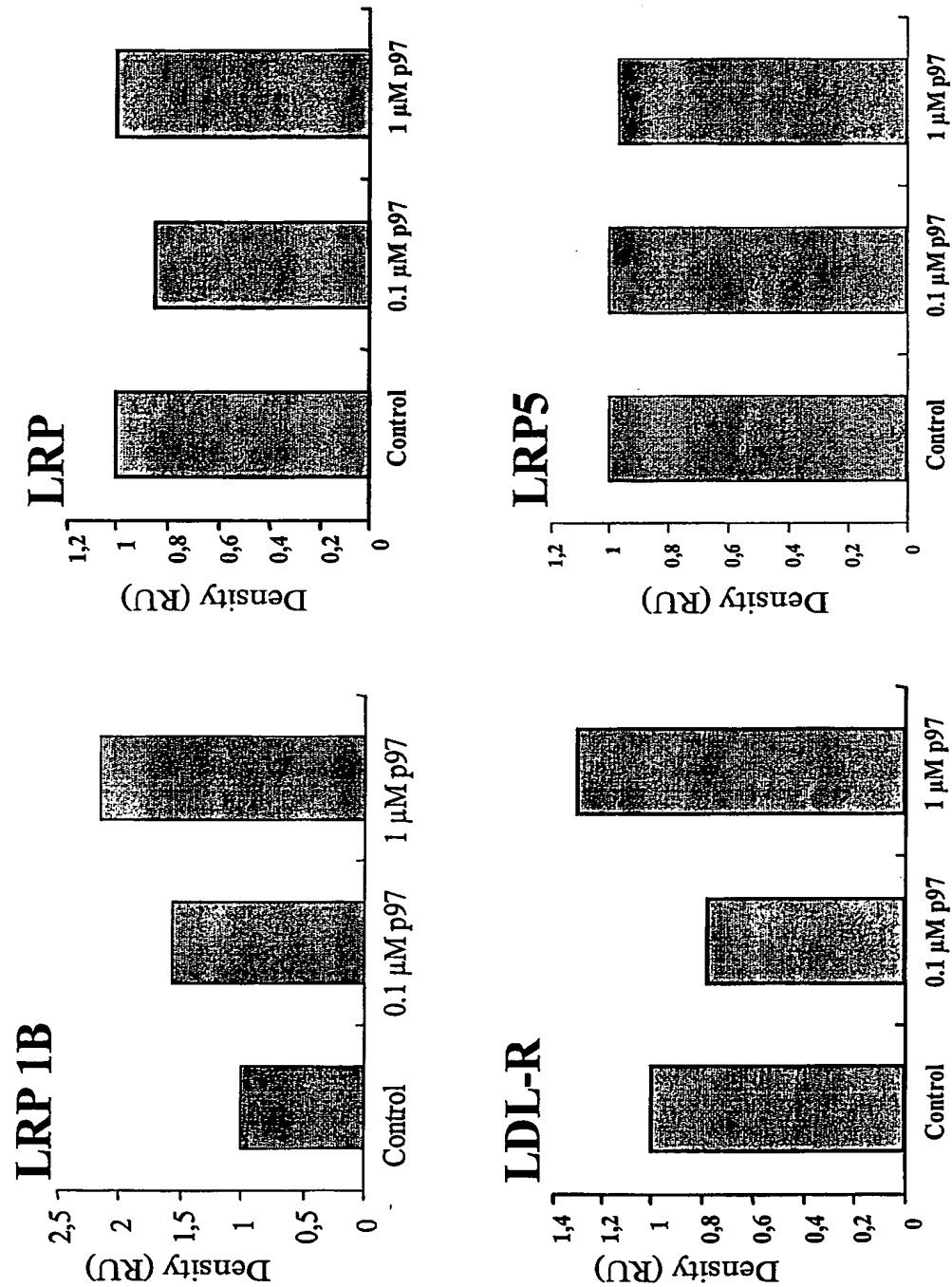


FIGURE 41

Members of LDL-receptor family in human endothelial cells

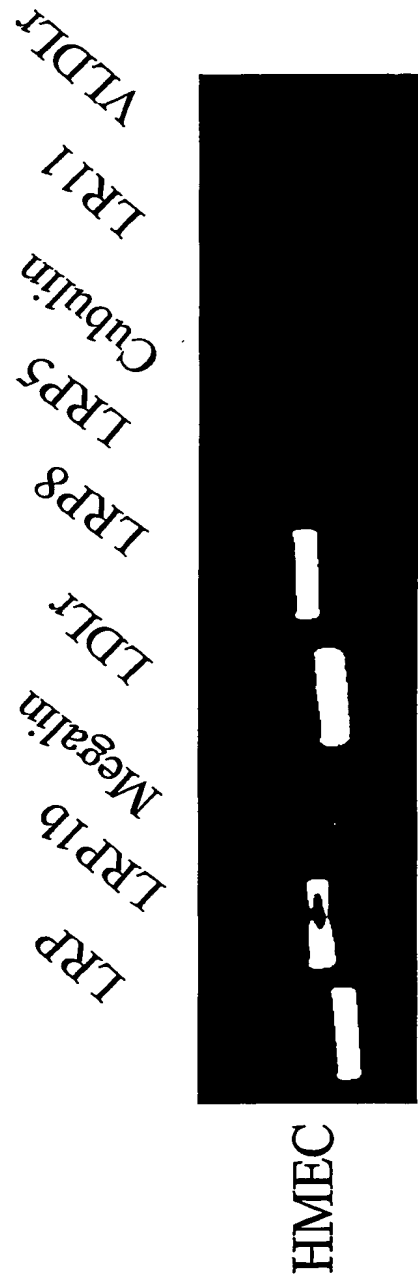
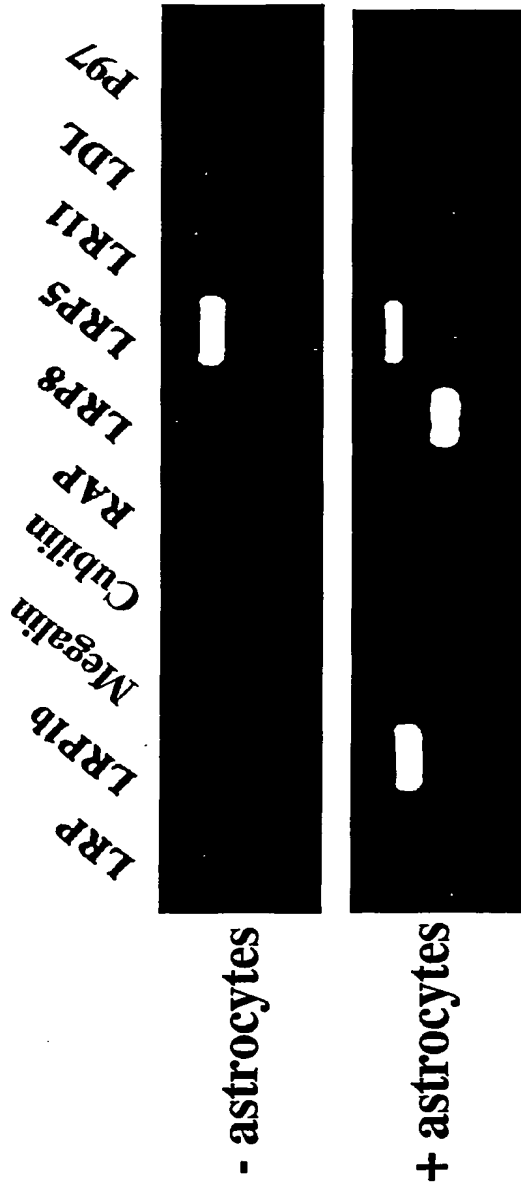


FIGURE 42

Expression of the LDL-receptor family in BBCE cells



- Human primers for LRP 1B, LRP8 and LRP5 can be used in bovine cells
- LRP 1B and LRP8 are up-regulated in co-culture

FIGURE 43

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22 July 2004 (22.07.2004)

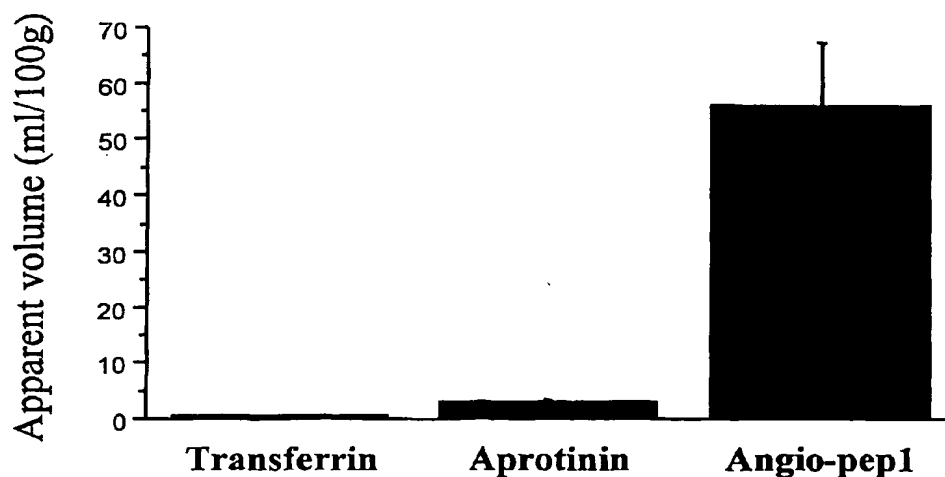
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60/437,986 6 January 2003 (06.01.2003) US
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- (72) Inventors; and
- (75) Inventors/Applicants (for US only): **BÉLIVEAU, Richard [CA/CA]**; 266 Wilson, H3E 1L8 Montréal, Québec, CA (CA). **DEMEULE, Michel [CA/CA]**; 3557 Archambault, J4M 2W8 Longueuil, Québec, CA (CA).
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- (84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).
- Published:
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[Continued on next page]

(54) Title: APROTININ AND ANGLOS AS CARRIERS ACROSS THE BLOOD-BRAIN BARRIER



(57) Abstract: The present invention relates to improvements in the field of drug delivery. More particularly, the invention relates to a non-invasive and flexible method and carrier for transporting a compound or drug across the blood-brain barrier of an individual. In particular the present invention relates to a carrier for transporting an agent attached thereto across a blood-brain barrier, wherein the carrier is able to cross the blood-brain barrier after attachment to the agent and thereby transport the agent across the blood-brain barrier. The present invention relates to improvements in the field of drug delivery. More particularly, the invention relates to a non-invasive and flexible method and carrier for transporting a compound or drug across the blood-brain barrier of an individual. In particular the present invention relates to a carrier for transporting an agent attached thereto across a blood-brain barrier, wherein the carrier is able to cross the blood-brain barrier after attachment to the agent and thereby transport the agent across the blood-brain barrier.



— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

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International Application No
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IPC 7 A61K47/48 A61P25/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS, CHEM ABS Data, EMBASE, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>DE 199 53 696 A (CHERKASKY ALEXANDER) 10 May 2001 (2001-05-10)</p> <p>column 1, lines 15-32; claim 3; figure 1 ----- -/--</p>	<p>1-4, 7-21, 24-38, 41-53, 56-67, 70-78, 80-102</p>

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

19 May 2004

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30. 09. 2004

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/CA2004/000011

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SHIMURA T ET AL: "TRANSPORT MECHANISM OF A NEW BEHAVIORALLY HIGHLY POTENT ADRENOCORTICOTROPIC HORMONE (ACTH) ANALOG, EBIRATIDE, THROUGH THE BLOOD-BRAIN BARRIER" JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS, AMERICAN SOCIETY FOR PHARMACOLOGY AND, US, vol. 258, no. 2, 1991, pages 459-465, XP008030272 ISSN: 0022-3565 abstract; figure 1	1,2,4, 7-19,21, 24-36, 38, 41-51, 53, 56-65, 67, 70-77, 80-102
Y	----- DEMEULE M ET AL: "HIGH TRANSCYTOSIS OF MELANOTRANSFERRIN (P97) ACROSS THE BLOOD-BRAIN BARRIER" JOURNAL OF NEUROCHEMISTRY, NEW YORK, NY, US, vol. 83, no. 4, November 2002 (2002-11), pages 924-933, XP001188983 ISSN: 0022-3042 see discussion abstract	1-5, 7-22, 24-39, 41-54, 56-68, 70-78, 80-102
Y	----- SEIDEL G ET AL: "EFFECTS OF TRASYLOL ON THE BLOOD-BRAIN BARRIER IN RATS" NAUNYN-SCHMIEDEBERG'S ARCHIVES OF PHARMACOLOGY, SPRINGER, BERLIN, DE, vol. 284, no. 4, 1974, page R73, XP008030270 ISSN: 0028-1298 abstract	1-5, 7-22, 24-39, 41-54, 56-68, 70-78, 80-102
Y	----- MARTEL ET AL: "Transport of apolipoproteins E and J at the blood - brain barrier. Relevance to Alzheimer's disease" STP PHARMA SCIENCES, PARIS, FR, vol. 7, no. 1, 1997, pages 28-36, XP002090769 ISSN: 1157-1489 abstract	1-5, 7-22, 24-39, 41-54, 56-68, 70-78, 80-102
P,X	----- WO 03/009815 A (KENNARD MALCOLM L ;YANG JOSEPH (CA); DEMEULE MICHEL (CA); BELIVEAU) 6 February 2003 (2003-02-06) page 4; figure 17 page 37, line 8; claims 8,25 -----	1-5, 7-22, 24-39, 41-54, 56-68, 70-78, 80-102

-/--

INTERNATIONAL SEARCH REPORT

International Application No

PCT/CA2004/000011

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>WO 02/33090 A (PROCYON BIOPHARMA INC) 25 April 2002 (2002-04-25)</p> <p>page 1, lines 42,43; claims 12,22,36,46,52,58,75,82,88; example 18</p>	<p>1-5, 7-22, 24-39, 41-54, 56-68, 70-78, 80-102</p>
A	<p>-----</p> <p>GUILLOT F L ET AL: "ANGIOTENSIN PEPTIDE REGULATION OF BOVINE BRAIN MICROVESSEL ENDOTHELIAL CELL MONOLAYER PERMEABILITY" JOURNAL OF CARDIOVASCULAR PHARMACOLOGY, NEW YORK, NY, US, vol. 18, no. 2, 1991, pages 212-218, XP008030278 ISSN: 0160-2446 abstract page 217, column 2</p>	<p>1-5, 7-22, 24-39, 41-54, 56-68, 70-78, 80-102</p>
P,Y	<p>-----</p> <p>KOBAYASHI H ET AL: "THE PROTEASE INHIBITOR BIKUNIN, A NOVEL ANTI-METASTATIC AGENT" BIOLOGICAL CHEMISTRY, XX, XX, vol. 384, no. 5, 1 May 2003 (2003-05-01), pages 749-754, XP008030275 ISSN: 1431-6730 abstract; figure 2</p> <p>-----</p>	<p>1-5, 7-22, 24-39, 41-54, 56-68, 70-78, 80-102</p>

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2004/000011

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-4, 7-21, 24-38, 41-53, 56-67, 70-78, 80-102 (all partially), 5, 22, 39
54, 68

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 5, 22, 39, 54, 68 complete; 1-4, 7-21, 24-38, 41-53, 56-67, 70-78, 80-102 partially

Carrier for transporting an agent attached thereto across the blood brain barrier wherein the agent is anticancer agent paclitaxel. Conjugate comprising the carrier and paclitaxel, pharmaceutical composition and use of the same for neurological disease (brain tumour, brain metastasis, schizophrenia, epilepsy, Alzheimer's disease, Parkinson's disease, Huntington's disease, stroke and obesity).

2. claims: 1-4, 6-21, 23-38, 40-53, 55-67, 69-79, 80-102 partially

Carrier for transporting an agent attached thereto across the blood brain barrier wherein the agent is a green fluorescent protein, a histag protein, and beta galactosidase. Conjugate comprising the carrier and the protein agent, pharmaceutical composition and use of the same for neurological disease (brain tumour, brain metastasis, schizophrenia, epilepsy, Alzheimer's disease, Parkinson's disease, Huntington's disease, stroke and obesity)

3. claims: 1-4, 6-21, 23-38, 40-53, 55-67, 69-79, 80-102 partially

Carrier for transporting an agent attached thereto across the blood brain barrier wherein the agent is a green fluorescent protein, a histag protein, and beta galactosidase. Conjugate comprising the carrier and the protein agent, pharmaceutical composition and use of the same for neurological disease (brain tumour, brain metastasis, schizophrenia, epilepsy, Alzheimer's disease, Parkinson's disease, Huntington's disease, stroke and obesity)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/CA2004/000011

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
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			WO 03009815 A2	06-02-2003
			US 2003129186 A1	10-07-2003

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			CA 2355334 A1	16-04-2002
			AU 1369102 A	29-04-2002
			WO 0233090 A2	25-04-2002
			CA 2359650 A1	16-04-2002
			EP 1326981 A2	16-07-2003
			JP 2004510833 T	08-04-2004
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GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE,
KG, KM, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA,
MD, MG, MK, MN, MW, MX, MZ, NA, NG, NI, NO, NZ,
OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL,
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European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI,
FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT,
RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA,
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ance Notes on Codes and Abbreviations" appearing at the begin-
ning of each regular issue of the PCT Gazette.*

(54) Title: APROTININ POLYPEPTIDES FOR TRANSPORTING A COMPOUND ACROSS THE BLOOD-BRAIN BARRIER

(57) Abstract: The invention relates to improvements in the field of drug delivery. More particularly, the invention relates to polypeptides derived from aprotinin and from aprotinin analogs as well as conjugates and pharmaceutical compositions comprising these polypeptides or conjugates. The present invention also relates to the use of these polypeptide for transporting a compound or drug across the blood-brain barrier of a mammal and in the treatment and diagnosis of neurological diseases.

WO 2006/086870 A1

MOLECULES FOR TRANSPORTING A COMPOUND ACROSS THE BLOOD-BRAIN BARRIER

FIELD OF THE INVENTION

5 The present invention relates to improvements in the field of drug delivery. More particularly, the invention relates to polypeptides, conjugates and pharmaceutical compositions comprising the polypeptides or conjugates of the present invention. The present invention also relates to the use of these polypeptides and
10 conjugates for transporting a compound or drug across the blood-brain barrier of a mammal and in the treatment and diagnosis of neurological diseases.

BACKGROUND OF THE INVENTION

In the development of a new therapy for brain pathologies, the blood-brain barrier
15 (BBB) is considered as a major obstacle for the potential use of drugs for treating disorders of the central nervous system (CNS). The global market for CNS drugs was \$33 billion in 1998, which was roughly half that of global market for cardiovascular drugs, even though in the United States, nearly twice as many people suffer from CNS disorders as from cardiovascular diseases. The reason
20 for this lopsidedness is that more than 98% of all potential CNS drugs do not cross the blood-brain barrier. In addition, more than 99% of worldwide CNS drug development is devoted solely to CNS drug discovery, and less than 1% is directed to CNS drug delivery. This ratio could explain why no efficient treatment is currently available for the major neurological diseases such as brain tumors,
25 Alzheimer's and stroke.

The brain is shielded against potentially toxic substances by the presence of two barrier systems: the blood-brain barrier (BBB) and the blood-cerebrospinal fluid barrier (BCSFB). The BBB is considered to be the major route for the uptake of serum ligands since its surface area is approximately 5000-fold greater than that
30 of BCSFB. The brain endothelium, which constitutes the BBB, represents the major obstacle for the use of potential drugs against many disorders of the CNS.

As a general rule, only lipophilic molecules smaller than about 500 Daltons may pass across the BBB, i.e., from blood to brain. However, the size of many drugs that show promising results in animal studies for treating CNS disorders is considerably bigger. Thus, peptide and protein therapeutics are generally
5 excluded from transport from blood to brain, owing to the negligible permeability of the brain capillary endothelial wall to these drugs. Brain capillary endothelial cells (BCECs) are closely sealed by tight junctions, possess few fenestrae and few endocytic vesicles as compared to capillaries of other organs. BCECs are surrounded by extracellular matrix, astrocytes, pericytes and microglial cells. The
10 close association of endothelial cells with the astrocyte foot processes and the basement membrane of capillaries are important for the development and maintenance of the BBB properties that permit tight control of blood-brain exchange.

International publication WO2004/060403 discloses an invention made by the
15 inventors relating to molecules for transporting a drug across the blood brain barrier. Otherwise, to date, there is no efficient drug delivery approach available for the brain. Methods under investigation for peptide and protein drug delivery to the brain may be divided in three principal strategies. Firstly, invasive procedures include the direct intraventricular administration of drugs by means of surgery, and
20 the temporary disruption of the BBB via intracarotid infusion of hyperosmolar solutions. Secondly, the pharmacologically-based strategy consists in facilitating the passage through the BBB by increasing the lipid solubility of peptides or proteins. Thirdly, physiologic-based strategies exploit the various carrier mechanisms at the BBB, which have been characterized in the recent years. In
25 this approach, drugs are attached to a protein vector that performs like receptors-targeted delivery vehicle on the BBB. This approach is highly specific and presents high efficacy with an extreme flexibility for clinical indications with unlimited targets. The latter approach has been, and is still, investigated by the inventors, who came up with the molecules described in the afore-mentioned
30 publication and those of the present invention.

U.S. patent no. 5,807,980 describes Bovine Pancreatic Trypsin Inhibitor (aprotinin) -derived inhibitors as well as a method for their preparation and therapeutic use. These peptides are used for the treatment of a condition

characterized by an abnormal appearance or amount of tissue factor and/or factor VIIIa such as abnormal thrombosis.

U.S. patent no. 5,780,265 describes serine protease inhibitors that are capable of inhibiting plasma kallikrein.

- 5 U.S. Patent no.5,118,668 describes Bovine Pancreatic Trypsin Inhibitor variants.

It would be highly desirable to be provided with improved molecules that can act as carriers or vectors for transporting a compound or drug across the BBB of an individual.

10 SUMMARY OF THE INVENTION

One aim of the present invention is to provide an improvement in the field of drug delivery.

- Another aim of the present invention is to provide a non-invasive and flexible method and carrier for transporting a compound or drug across the blood-brain barrier of an individual.

The present application discloses new molecules which may be able, for example, of transporting desirable compounds across the blood brain barrier.

- In a first aspect the present invention provides a biologically active polypeptide which may be able to cross (i.e., crossing) a cell layer mimicking (which mimics) a mammalian blood brain barrier in an *in vitro* assay, the polypeptide may be selected, for example, from the group of

- aprotinin (SEQ ID NO.:98),
- an aprotinin analogue
- an aprotinin fragment which may comprise (or may consist essentially of) the amino acid sequence defined in SEQ ID NO.:1,
- a biologically active analogue of SEQ ID NO.:1,
- a biologically active fragment of SEQ ID NO.:1, and;
- a biologically active fragment of a SEQ ID NO.:1 analogue.

In a second aspect the present invention provides, a biologically active polypeptide which may be able to cross (i.e., crossing) a cell layer mimicking (which mimics) a mammalian blood brain barrier in an *in vitro* assay, the

5 polypeptide may be selected, for example, from the group of;

- an aprotinin fragment which may comprise the amino acid sequence defined in SEQ ID NO.:1,
- a biologically active analogue of SEQ ID NO.:1,
- a biologically active fragment of SEQ ID NO.:1 and;
- 10 - a biologically active fragment of a SEQ ID NO.:1 analogue.

In accordance with the present invention the aprotinin fragment may consist of the sequence defined in SEQ ID NO.:1. Further in accordance with the present invention, the aprotinin fragment may comprise SEQ ID NO.1 and may have a
15 length of from about 19 amino acids to about 54 amino acids, e.g., from 10 to 50 amino acids in length, from 10 to 30 amino acids in length etc.

In accordance with the present invention, the biologically active analogue of SEQ ID NO.:1, may have a length of from about 19 amino acids to about 54 amino
20 acids (e.g., including for example 21 to 23, 25 to 34, 36 to 50 and 52 to 54), or of from about 19 amino acids to about 50 amino acids, or from about 19 amino acids to about 34 amino acids (e.g., 19, 20, 21, 22, 23, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34), of from about 19 amino acids to about 23 amino acids or of about 19, 20, 21, 22, 23, 24, 35, 51, amino acids.

25 A biologically active fragment of a polypeptide (e.g., of 19 amino acids) described herein may include for example a polypeptide of from about 7, 8, 9 or 10 to 18 amino acids. Therefore, in accordance with the present invention, a biologically active fragment of SEQ ID NO.:1 or of a SEQ ID NO.:1 analogue may have a
30 length of from about 7 to about 18 amino acids or from about 10 to about 18.

U.S. patent no. 5,807,980 describes a polypeptide which is identified herein as SEQ ID NO.:102.

U.S. Patent no. 5,780,265 describes a polypeptide which is identified herein as SEQ ID NO.:103.

The aprotinin amino acid sequence (SEQ ID NO.:98), the Angiopep-1 amino acid sequence (SEQ ID NO.:67), as well as some sequences of biologically active
5 analogs may be found for example in international application no. PCT/CA2004/000011 published on July 22, 2004 in under international publication no. WO2004/060403. Additionally, international publication No. WO04/060403 describes a polypeptide which is identified herein as SEQ ID NO.: 104.

10 U.S. Patent no.5,118,668 describes polypeptides which has the sequence illustrated in SEQ ID NO.: 105.

Examples of aprotinin analogs may be found by performing a protein blast (Genebank: www.ncbi.nlm.nih.gov/BLAST/) of the synthetic aprotinin sequence
15 (or portion thereof) disclosed in international application no. PCT/CA2004/000011. Exemplary aprotinin analogs may be found, for example under accession nos. CAA37967 (GI:58005), 1405218C (GI:3604747) etc.

In a further aspect the present invention provides a biologically active polypeptide
20 which may be able to cross (i.e., crossing) a cell layer mimicking (which mimics) a mammalian blood brain barrier in an *in vitro* assay, the polypeptide may be selected, for example, from the group of;

- an aprotinin fragment of from 19 to 54 (e.g., 19-50) amino acid long, which may comprise SEQ ID NO.:1,
- 25 - an aprotinin fragment consisting of SEQ ID NO.:1,
- a biologically active analogue of SEQ ID NO.:1 of from about 19 to 50 amino acids long, and;
- a biologically active fragment of SEQ ID NO.:1 (of from 10 to 18 amino acids) or biologically active fragment of a SEQ ID NO.:1 analogue (of
30 from about 10 to 18 amino acids).

In accordance with the present invention there is provided a biologically active analogue of SEQ ID NO.:1 which may be selected, for example, from the group consisting of

- 5 - a SEQ ID NO.:1 analogue which may comprise at least 35 % identity with the amino acid sequence of SEQ ID NO.:1,
- a SEQ ID NO.:1 analogue which may comprise at least 40 % identity with the amino acid sequence of SEQ ID NO.:1,
- a SEQ ID NO.:1 analogue which may comprise at least 50 % identity with the amino acid sequence of SEQ ID NO.:1,
- 10 - a SEQ ID NO.:1 analogue which may comprise at least 60 % identity with the amino acid sequence of SEQ ID NO.:1,
- a SEQ ID NO.:1 analogue which may comprise at least 70 % identity with the amino acid sequence of SEQ ID NO.:1,
- a SEQ ID NO.:1 analogue which may comprise at least 80 % identity with the amino acid sequence of SEQ ID NO.:1,
- 15 - a SEQ ID NO.:1 analogue which may comprise at least 90 % identity with the amino acid sequence of SEQ ID NO.:1 and;
- a SEQ ID NO.:1 analogue which may comprise at least 95 % (i.e., 96%, 97%, 98%, 99% and 100%) identity with the amino acid
- 20 sequence of SEQ ID NO.:1.

For example, the biologically active analogue of SEQ ID NO.:1 may comprise an amino acid sequence selected from the group consisting of an amino acid sequence defined in any one of SEQ ID NO.:2 to SEQ ID NO.: 62, SEQ ID NO.: 25 68 to SEQ ID NO.: 93, and SEQ ID NO.:97 as well as 99, 100 and 101. When the polypeptide of the present invention comprises, for example, SEQ ID NO.:99, 100 or 101, the polypeptide may have an amino acid sequence of from about 10 to 50 amino acids, e.g., from 10 to 30 amino acids in length.

30 Further in accordance with the present invention, the biologically active analogue of SEQ ID NO.:1 may comprise the amino acid sequence defined in SEQ ID NO.:67 (i.e., polypeptide no. 67 which is an amidated version of SEQ ID NO.:67 (Angiopep-1)).

The polypeptides of the present invention may be amidated, i.e., may have an amidated amino acid sequence. For example, the polypeptide of SEQ ID NO.:67 may be amidated (polypeptide no.67).

5 Portion of the present invention may relate to the polypeptides defined herein with the exception of polypeptides defined in SEQ ID NO.: 102, 103, 104 and 105, while other portion of the invention may include these peptides. For example and without limitation, conjugates comprising these peptides as well as their use for treating a neurological disease (e.g., brain tumor), method of treatment of a
10 neurological disease (e.g., brain tumor), pharmaceutical composition for treating a neurological disease, etc. are encompassed by the present invention.

In yet a further aspect the present invention provides a biologically active polypeptide which may be able to cross (i.e., crossing) a cell layer mimicking
15 (which mimics) a mammalian blood brain barrier in an *in vitro* assay, the polypeptide may be selected, for example, from the group of;

- an aprotinin fragment of from 19 to 54 (e.g., 19-50) amino acid long, which may comprise SEQ ID NO.:1,
- an aprotinin fragment consisting of SEQ ID NO.:1,
- 20 - a biologically active analogue of SEQ ID NO.:1 of from about 19 to 50 amino acids long, provided that said analogue does not comprise SEQ ID NO.: 102, 103, 104 or 105 and provided that when said analogue consists of SEQ ID NO.:67 said analogue is amidated,
- a biologically active fragment of SEQ ID NO.:1 of from 10 to 18 amino
25 acids, and;
- a biologically active fragment of a SEQ ID NO.:1 analogue of from about 10 to 18 amino acids.

Further in accordance with the present invention, the biologically active fragment
30 of SEQ ID NO.:1 or the biologically active fragment of a SEQ ID NO.:1 analogue may comprise at least 9 or at least 10 (consecutive or contiguous) amino acids of SEQ ID NO.1 or of the SEQ ID NO.:1 analogue.

The polypeptides of the present invention may have an amino acid sequence which may comprise of from between 1 to 12 amino acid substitutions (i.e., SEQ ID NO.:91). For example, the amino acid substitution may be from between 1 to 10 amino acid substitutions, or from 1 to 5 amino acid substitutions. In accordance with the present invention, the amino acid substitution may be a non-conservative amino acid substitution or a conservative amino acid substitution.

For example, when a polypeptide of the present invention comprises amino acids which are identical to those of SEQ ID NO.:1 and other amino acids which are not identical (non-identical), those which are non-identical may be a conservative amino acid substitution. The comparison of identical and non-identical amino acids may be performed by looking at a corresponding location.

Examples of SEQ ID NO.:1 analogue which may have at least 35% identity includes for example, a polypeptide comprising (consisting of) the amino acid sequence defined in SEQ ID NO.:91 (about 36.8% identity, i.e., 7 amino acid out of 19 amino acids of SEQ ID NO.:91 are identical to SEQ ID NO.:1), a polypeptide comprising (consisting of) the amino acid sequence defined in SEQ ID NO.:98 (about 68.4% identity, i.e., 13 amino acid out of 19 amino acids are identical to SEQ ID NO.:1), a polypeptide comprising (consisting of) the amino acid sequence defined in SEQ ID NO.:67 (about 73.7% identity, i.e., 14 amino acid out of 19 amino acids are identical to SEQ ID NO.:1), a polypeptide comprising (consisting of) the amino acid sequence defined in SEQ ID NO.: 76 (about 73.7% identity, i.e., 14 amino acid out of 19 amino acids are identical to SEQ ID NO.:1) and a polypeptide comprising (consisting of) the amino acid sequence defined in SEQ ID NO.:5 (about 79 % identity, i.e., 15 amino acid out of 19 amino acids are identical to SEQ ID NO.:1).

Examples of SEQ ID NO.:1 analogue which may have at least 60% identity includes for example, a polypeptide comprising (consisting of) the amino acid sequence defined in SEQ ID NO.:98 (about 68.4% identity, i.e., 13 amino acid out of 19 amino acids are identical to SEQ ID NO.:1), a polypeptide comprising (consisting of) the amino acid sequence defined in SEQ ID NO.:67 (about 73.7% identity, i.e., 14 amino acid out of 19 amino acids are identical to SEQ ID NO.:1), a

polypeptide comprising (consisting of) the amino acid sequence defined in SEQ ID NO.: 76 (about 73.7% identity, i.e., 14 amino acid out of 19 amino acids are identical to SEQ ID NO.:1) and a polypeptide comprising (consisting of) the amino acid sequence defined in SEQ ID NO.:5 (about 79 % identity, i.e., 15 amino acid out of 19 amino acids are identical to SEQ ID NO.:1).

Examples of SEQ ID NO.:1 analogue which may have at least 70% identity includes for example, a polypeptide comprising (consisting of) the amino acid sequence defined in SEQ ID NO.:67 (about 73.7% identity, i.e., 14 amino acid out of 19 amino acids are identical to SEQ ID NO.:1), SEQ ID NO.: 76 (about 73.7% identity, i.e., 14 amino acid out of 19 amino acids are identical to SEQ ID NO.:1), SEQ ID NO.:5 (about 79 % identity, i.e., 15 amino acid out of 19 amino acids are identical to SEQ ID NO.:1).

In accordance, with the present invention, the carrier may more particularly be selected from the group consisting of peptide Nos. 5, 67, 76, 91 and peptide 97 (i.e., SEQ ID NO.:5, 67, 76, 91 and 97 (Angiopep-2)). The carrier may be used, for example, for transporting an agent attached thereto across a blood-brain barrier. In accordance with the present invention, the carrier may be able to cross the blood-brain barrier after attachment to the agent and may therefore be able to transport the agent across the blood-brain barrier.

In accordance with the present invention, the polypeptides may be in an isolated form or in a substantially purified form.

More particularly, the present invention provides a carrier for transporting an agent attached thereto across a blood-brain barrier, wherein the carrier may be able to cross the blood-brain barrier after attachment to the agent and thereby transport the agent across the blood-brain barrier. The carrier may comprise at least one polypeptide of the present invention (provided that when said polypeptide or carrier consist of SEQ ID NO.:67, said polypeptide is modified by a group e.g., amidated). For example, the carrier may be selected from a class of molecules related to aprotinin.

The transporting activity which is effected by the carrier does not affect blood-brain barrier integrity. The transporting of an agent may result, for example, in the delivery of the agent to the central nervous system (CNS) of an individual.

It is to be understood herein that the polypeptides of the present invention may be synthesized chemically (e.g., solid phase synthesis) or may be produced by recombinant DNA technology. Codons which encode specific amino acids are well known in the art and is discuss, for example, in Biochemistry (third edition; 1988, Lubert Stryer, Stanford University, W.H. Freeman and Company, New-York). A nucleotide sequence encoding a carrier of the present invention is therefore encompassed herein. More particularly, nucleotide sequences (deoxyribonucleotides or ribonucleotides or derivatives thereof) encoding a polypeptide selected from the group consisting of any one of SEQ ID NO.:1 to 97, are encompassed by the present invention. An exemplary nucleotide sequence encoding an aprotinin analogue is illustrated in SEQ ID NO.:106 and may be found in Gene Bank under accession no.X04666. This sequence encodes an aprotinin analogue having a lysine at position 16 (with reference to the amino acid sequence encoded by SEQ ID NO.:106) instead of a valine as found in SEQ ID NO.:98. A mutation in the nucleotide sequence of SEQ ID NO.:106 may be introduced by methods known in the art to change the produce the peptide of SEQ ID NO.:98 having a valine in position 16. Techniques known in the art may be used to introduce further mutations in the nucleotide sequence to encode analogues of the present invention. Fragments may be obtained from this nucleotide sequence by enzymatic digestion or polymerase chain reaction, etc. Alternatively, a desired nucleotide sequence may be synthesized chemically by methods known in the art.

In a further aspect, the present invention relates to a conjugate which may comprise a carrier selected from the group consisting of any one of the polypeptide of the present invention, and an agent selected from the group consisting, for example, of a drug (e.g., a small molecule drug, e.g., an antibiotic), a medicine, a detectable label, a protein (e.g., an enzyme), protein-based compound (e.g., a protein complex comprising one or polypeptide chain) and a polypeptide (peptide). The agent may be more particularly, a molecule which is

active at the level of the central nervous system. The agent may be any agent for treating or detecting a neurological disease.

In accordance with the present invention the carrier which is part of conjugate may be selected, for example, from the group of;

- 5 - an aprotinin fragment of from 10 to 54 (e.g., 19-50) amino acid long, which may comprise SEQ ID NO.:1,
- an aprotinin fragment consisting of SEQ ID NO.:1,
- a biologically active analogue of SEQ ID NO.:1 (e.g., of from about 19 to 50 amino acids long), provided that when said analogue consists of
- 10 SEQ ID NO.:67 said analogue is amidated,
- a biologically active fragment of SEQ ID NO.:1 of from 10 to 18 amino acids, and;
- biologically active fragment of a SEQ ID NO.:1 analogue of from about 10 to 18 amino acids.

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In accordance with the present invention, the agent may have a maximum molecular weight of about 160,000 Daltons.

20 Further in accordance with the present invention, the transporting activity may be effected by receptor-mediated transcytosis or adsorptive-mediated transcytosis. The agent may be one able to be transported by such mechanism.

25 Further in accordance with the present invention, the conjugate may be in the form of a fusion protein which may have a first moiety consisting essentially of the carrier of the present invention and a second moiety consisting essentially of a protein or protein-based agent.

30 Exemplary neurological diseases which may be treated or detected by the carrier and/or conjugate is a disease selected, for example, from the group consisting of a brain tumor, a brain metastasis, schizophrenia, epilepsy, Alzheimer's disease, Parkinson's disease, Huntington's disease, stroke and blood-brain barrier related malfunctions (e.g., obesity).

In accordance with the present invention, the blood-brain barrier related malfunction is obesity. Also in accordance with the present invention, the agent which may be conjugated with the carrier of the present invention may be a leptin. A conjugate comprising a leptin and a carrier may be used, for example, in the treatment of obesity.

In accordance with the present invention, the detectable label may be a radioimaging agent. Example of a label which may be conjugated with the carrier of the present invention and which is encompassed herein includes, for example and without limitation, an isotope, a fluorescent label (e.g., rhodamine), a reporter molecule (e.g., biotin), etc. Other examples of detectable labels include, for example, a green fluorescent protein, biotin, a histag protein and β -galactosidase.

Example of a protein or protein-based compound which may be conjugated with the carrier of the present invention and which is encompassed herein includes, without limitation, an antibody, an antibody fragment (e.g., an antibody binding fragment such as Fv fragment, F(ab)2, F(ab)2' and Fab and the like), a peptidic or protein-based drug (e.g., a positive pharmacological modulator (agonist) or an pharmacological inhibitor (antagonist)) etc. Other examples of agent which are encompassed herein include cellular toxins (e.g., monomethyl auristatin E (MMAE), toxins from bacteria endotoxins and exotoxins; diphtheria toxins, botulinum toxins, tetanus toxins, pertussis toxins, staphylococcus enterotoxins, toxin shock syndrome toxin TSST-1, adenylate cyclase toxin, shiga toxin, cholera enterotoxin, and others) and anti-angiogenic compounds (endostatin, catechins, nutraceuticals, chemokine IP-10, inhibitors of matrix metalloproteinase (MMPs), anastellin, vironectin, antithrombin, tyrosine kinase inhibitors, VEGF inhibitors, antibodies against receptor, herceptin, avastin and panitumumab and others).

Also in accordance with the present invention, the agent may be a small molecule drug such as an anticancer drug (e.g., for treating a brain tumor). An anticancer drug encompassed by the present invention may include, for example, a drug having a group allowing its conjugation to the carrier of the present invention. Examples of anticancer drug includes, for example, without limitation, a drug which may be selected from the group consisting of paclitaxel (Taxol), vinblastine,

vincristine, etoposide, doxorubicin, cyclophosphamide, taxotere, melphalan, chlorambucil, and any combination.

More particularly, the conjugate of the present invention may comprise the formula
5 R-L-M or pharmaceutically acceptable salts thereof, wherein R is a class of molecules related to aprotinin (e.g., aprotinin, aprotinin fragment, Angiopep-1, Angiopep-2, analogs, derivatives or fragments). For example, R may be a carrier selected from a class of molecules related to aprotinin able to cross the blood-brain barrier after attachment to L-M and thereby transport M across the blood-
10 brain barrier. L may be a linker or a bond (chemical bond). M may be an agent selected from the group consisting of a drug (e.g., a small molecule drug), a medicine, a (detectable) label, a protein or protein-based compound (e.g., antibody, an antibody fragment), an antibiotic, an anti-cancer agent, an anti-angiogenic compound and a polypeptide or any molecule active at the level of the
15 central nervous system. It is to be understood herein that the formula R-L-M is not intended to be restricted to a specific order or specific ratio. As being exemplified herein, M may be found in several ratios over R.

For example, conjugates of formula R-L-M or a pharmaceutically acceptable salt
20 thereof, may be used for transporting M across a blood-brain barrier, where R may be for example, a carrier selected from the group consisting of peptide Nos: 5, 67, 76, 91 and 97 as described in herein. The carrier may be able to cross the blood-brain barrier after attachment to L-M and may therefore transport M across the blood-brain barrier.

25 In accordance with the present invention, M may be an agent useful for treating or diagnosing a neurological disease.

It is to be understood herein that when more than one carrier conjugation site are
30 available or present, more than one drug or drug molecule may be conjugated to the carrier of the present invention. Therefore, the conjugate may comprise one or more drug molecules. The conjugate may be active by itself, i.e., the drug may be active even when associated with the carrier. Also in accordance with the present invention, the compound may or may not be released from the carrier i.e.,

generally after transport across the blood-brain barrier. The compound may therefore be releasable from the conjugate (or from the carrier) and may become active thereafter. More particularly, the agent may be releasable from the carrier after transport across the blood-brain barrier.

- 5 In accordance with another embodiment of the present invention, there is provided a conjugate for transporting an agent across a blood-brain barrier, the conjugate may comprise: (a) a carrier; and (b) an agent attached to the carrier, wherein the conjugate is able to cross the blood-brain barrier and thereby transport the agent across the blood-brain barrier.
- 10 In a further aspect, the present invention relates to the use of a carrier or a conjugate of the present invention for transporting an agent across a blood brain barrier of a mammal in need thereof.

- 15 In yet a further aspect, the present invention relates to the use of a class of molecules related to aprotinin for transporting a compound attached thereto across the blood-brain barrier of a patient.

- In an additional aspect, the present invention relates to the use of a carrier or a conjugate as described herein for the diagnosis of a neurological disease or a central nervous system disease. For example, the carrier or conjugate may be
- 20 used for the *in vivo* detection of a neurological disease.

The carrier may be selected, for example, from the group of (biologically active);

- aprotinin (SEQ ID NO.:98),
- an aprotinin fragment which may comprise the amino acid sequence
- 25 defined in SEQ ID NO.:1,
- an aprotinin fragment consisting of SEQ ID NO.:1,
- a biologically active analogue of SEQ ID NO.:1, and;
- a biologically active fragment of SEQ ID NO.:1 or biologically active fragment of a SEQ ID NO.:1 analogue.

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More particularly, the carrier may be selected, for example, from the group of (biologically active);

- 15 -

- an aprotinin fragment which may comprise the amino acid sequence defined in SEQ ID NO.:1,
- an aprotinin fragment consisting of SEQ ID NO.:1,
- a biologically active analogue of SEQ ID NO.:1, and;
- 5 - a biologically active fragment of SEQ ID NO.:1 or biologically active fragment of a SEQ ID NO.:1 analogue.

In accordance with the present invention, when that analogue consists of SEQ ID NO.:67, said analogue is amidated.

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Even more particularly, the carrier may be selected, for example, from the group of;

- an aprotinin fragment of from 10 to 54 (e.g., 19-50) amino acid long, which may comprise SEQ ID NO.:1,
- 15 - an aprotinin fragment consisting of SEQ ID NO.:1,
- a biologically active analogue of SEQ ID NO.:1 (e.g., of from about 19 to 50 amino acids long), provided that when said analogue consists of SEQ ID NO.:67, said analogue is amidated,
- a biologically active fragment of SEQ ID NO.:1 of from 10 to 18 amino acids, and;
- 20 - a biologically active fragment of a SEQ ID NO.:1 analogue of from about 10 to 18 amino acids.

In another aspect, the present invention relates to the use of a class of molecules related to aprotinin in the manufacture of a medicament.

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According to the present invention, there is provided the use of a class of molecules related to aprotinin in the manufacture of a medicament for treating a neurological disease, or for treating a central nervous system disorder.

In yet another aspect, the present invention relate to the use of a carrier or conjugate described herein, in the manufacture of a medicament for treating a brain disease (a brain-associated disease) or neurological disease, for the

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diagnosis of a brain disease or neurological disease or for transporting an agent across the blood-brain barrier

In an additional aspect, the present invention relates to the use of a carrier or conjugate of the present invention for treating a mammal having, for example, a neurological disease or for the diagnosis of a neurological disease in a mammal in need thereof.

In accordance with the present invention, neurological disease encompassed by the present invention includes, for example and without limitation, a brain tumor, a brain metastasis, schizophrenia, epilepsy, Alzheimer's disease, Parkinson's disease, Huntington's disease, stroke and blood-brain barrier related malfunctions.

In a further aspect, the present invention relates to a method for transporting an agent across the blood-brain barrier of a mammal (human, animal), which may comprise the step of administering to the mammal a compound comprising the agent attached to a class of molecules related to aprotinin.

In yet a further aspect, the present invention provides a method for treating a neurological disease of a patient comprising administering to the patient a medicament comprising a class of molecules related to aprotinin, and a compound adapted to treat the disease, the compound being attached to the class of molecules related to aprotinin.

In an additional aspect, there is provided a method for treating a central nervous system disorder of a patient comprising administering to the patient a medicament comprising a class of molecules related to aprotinin, and a compound adapted to treat the disease, the compound being attached to the aprotinin.

In yet an additional aspect there is provided a method for transporting an agent across a blood-brain barrier, which comprises the step of administering to an individual a pharmaceutical composition of the present invention.

The present invention also relates, in a further aspect to a method for treating a mammal (e.g., a patient) in need thereof (e.g., a patient having a neurological disease). The method may comprise administering a carrier, a conjugate and/or a pharmaceutical composition of the present invention to the mammal.

The present invention additionally relates to a method for (of) diagnosing (i.e., a diagnostic method) a neurological disease in a mammal (e.g., a patient) in need thereof. The method may comprise administering a carrier, a conjugate and/or a pharmaceutical composition of the present invention to the mammal (human individual, patient, animal).

In accordance with the present invention, the administration may be performed intra-arterially, intra-nasally, intra-peritoneally, intravenously, intramuscularly, subcutaneously, transdermally or *per os*.

In accordance with the present invention, the pharmaceutical composition may be administered to the mammal in a therapeutically effective amount.

A mammal in need (individual in need) may be, for example, a mammal which has or is at risk of having a neurological disease, a central nervous system disease, brain cancer, a brain metastasis, etc.

In an additional aspect, the present invention relates to a pharmaceutical composition which may comprise, for example;

- a carrier (which may be selected from the group consisting of any of the polypeptide described herein) or conjugate of the present invention; and
- a pharmaceutically acceptable carrier, e.g., a pharmaceutically acceptable excipient.

In accordance with the present invention, the pharmaceutical composition may be used, for example, for the treatment of a neurological disease.

Further in accordance with the present invention, the pharmaceutical composition may be used, for example, for the diagnosis of a neurological disease.

Also in accordance with the present invention, the pharmaceutical composition may be used for example, for transporting an agent across a blood-brain barrier.

Also in accordance with the present invention, the pharmaceutical composition may be used for example, for the delivery of an agent to the CNS of an individual.

- 5 Further in accordance with the present invention, the pharmaceutical composition may be used for example, for treating a central nervous system disorder of a mammal in need thereof

- 10 In accordance with the present invention, pharmaceutical composition may be used for delivery of an agent to the CNS of an individual

It is to be understood herein that a pharmaceutically acceptable salts of a carrier (polypeptide) or of a conjugate is encompassed by the present invention.

- 15 The composition (pharmaceutical composition) may thus comprise a medicament manufactured as defined herein in association with a pharmaceutically acceptable excipient.

For the purpose of the present invention the following terms are defined below.

- 20 The term "carrier" or "vector" is intended to mean a compound or molecule such as a polypeptide that is able to transport a compound. For example, transport may occur across the blood-brain barrier. The carrier may be attached to (covalently or not) or conjugated to another compound or agent and thereby may be able to transport the other compound or agent across the blood-brain barrier. For example, the carrier may bind to receptors present on brain endothelial cells and thereby be transported across the blood-brain barrier by transcytosis. The carrier may be a molecule for which high levels of transendothelial transport may be obtained, without affecting the blood-brain barrier integrity. The carrier may be, but is not limited to, a protein, a peptide or a peptidomimetic and may be naturally occurring or produced by chemical synthesis or recombinant genetic technology (genetic engineering).
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The term "conjugate" is intended to mean a combination of a carrier and another compound or agent. The conjugation may be chemical in nature, such as via a

linker, or genetic in nature for example by recombinant genetic technology, such as in a fusion protein with for example a reporter molecule (e.g. green fluorescent protein, β -galactosidase, Histag, etc.).

5 The expression "small molecule drug" is intended to mean a drug having a molecular weight of 1000 g/mol or less.

10 The terms "treatment", "treating" and the like are intended to mean obtaining a desired pharmacologic and/or physiologic effect, e.g., inhibition of cancer cell growth, death of a cancer cell or amelioration of a neurological disease or condition. The effect may be prophylactic in terms of completely or partially preventing a disease or symptom thereof and/or may be therapeutic in terms of a partial or complete cure for a disease and/or adverse effect attributable to the disease. "Treatment" as used herein covers any treatment of a disease in a mammal, particularly a human, and includes: (a) preventing a disease or condition (e.g., preventing cancer) from occurring in an individual who may be predisposed to the disease but has not yet been diagnosed as having it; (b) inhibiting a disease, (e.g., arresting its development); or (c) relieving a disease (e.g., reducing symptoms associated with a disease). "Treatment" as used herein covers any administration of a pharmaceutical agent or compound to an individual to treat, cure, alleviate, improve, diminish or inhibit a condition in the individual, including, 20 without limitation, administering a carrier-agent conjugate to an individual.

25 The term "cancer" is intended to mean any cellular malignancy whose unique trait is the loss of normal controls which results in unregulated growth, lack of differentiation and ability to invade local tissues and metastasize. Cancer can develop in any tissue of any organ. More specifically, cancer is intended to include, without limitation, cancer of the brain.

30 The term "administering" and "administration" is intended to mean a mode of delivery including, without limitation, intra-arterially, intra-nasally, intra-peritoneally, intravenously, intramuscularly, sub-cutaneously, transdermally or *per os*. A daily dosage can be divided into one, two or more doses in a suitable form to be administered at one, two or more times throughout a time period.

The term "therapeutically effective" or "effective amount" is intended to mean an amount of a compound sufficient to substantially improve some symptom associated with a disease or a medical condition. For example, in the treatment of cancer or a mental condition or neurological or CNS disease, an agent or compound which decreases, prevents, delays, suppresses, or arrests any symptom of the disease or condition would be therapeutically effective. A therapeutically effective amount of an agent or compound is not required to cure a disease or condition but will provide a treatment for a disease or condition such that the onset of the disease or condition is delayed, hindered, or prevented, or the disease or condition symptoms are ameliorated, or the term of the disease or condition is changed or, for example, is less severe or recovery is accelerated in an individual.

The carrier and conjugates of the present invention may be used in combination with either conventional methods of treatment and/or therapy or may be used separately from conventional methods of treatment and/or therapy.

When the conjugates of this invention are administered in combination therapies with other agents, they may be administered sequentially or concurrently to an individual. Alternatively, pharmaceutical compositions according to the present invention may be comprised of a combination of a carrier-agent conjugate of the present invention in association with a pharmaceutically acceptable excipient, as described herein, and another therapeutic or prophylactic agent known in the art.

Pharmaceutically acceptable acid addition salts may be prepared by methods known and used in the art and are encompassed by the present invention.

Biologically active polypeptides of the present invention encompass functional derivatives. The term "functional derivative" is intended to mean a "chemical derivative", "fragment", or "variant" biologically active sequence or portion of a carrier or agent or conjugate and a salt thereof of the present invention. A carrier functional derivative may be able to be attached to or conjugated to another compound or agent and cross the blood-brain barrier and thereby be able to transport the other compound or agent across the blood-brain barrier.

The term "chemical derivative" is intended to mean a carrier, an agent, or a conjugate of the present invention, which contains additional chemical moieties not a part of the carrier, agent or carrier-agent conjugate. Covalent modifications are included within the scope of this invention. A chemical derivative may be conveniently prepared by direct chemical synthesis, using methods well known in the art. Such modifications may be, for example, introduced into a protein or peptide carrier, agent or carrier-agent conjugate by reacting targeted amino acid residues with an organic derivatizing agent that is capable of reacting with selected side chains or terminal residues. A carrier chemical derivative is able to cross the blood-brain barrier and be attached to or conjugated to another compound or agent and thereby be able to transport the other compound or agent across the blood-brain barrier. In a preferred embodiment, very high levels of transendothelial transport across the blood-brain barrier are obtained without any effects on the blood-brain barrier integrity.

The term "agent" is intended to mean without distinction an antibody, a drug (such as a medicinal drug) or a compound such as a therapeutic agent or compound, a marker, a tracer or an imaging compound.

The term "therapeutic agent" or "agent" is intended to mean an agent and/or medicine and/or drug used to treat the symptoms of a disease, physical or mental condition, injury or infection and includes, but is not limited to, antibiotics, anti-cancer agents, anti-angiogenic agents and molecules active at the level of the central nervous system. Paclitaxel, for example, can be administered intravenously to treat brain cancer.

The term "condition" is intended to mean any situation causing pain, discomfort, sickness, disease or disability (mental or physical) to or in an individual, including neurological disease, injury, infection, or chronic or acute pain. Neurological diseases which can be treated with the present invention include, but are not limited to, brain tumors, brain metastases, schizophrenia, epilepsy, Alzheimer's disease, Parkinson's disease, Huntington's disease and stroke.

As used herein, "pharmaceutical composition" means therapeutically effective amounts of the agent together with pharmaceutically acceptable diluents,

preservatives, solubilizers, emulsifiers, adjuvant and/or carriers. A "therapeutically effective amount" as used herein refers to that amount which provides a therapeutic effect for a given condition and administration regimen. Such compositions are liquids or lyophilized or otherwise dried formulations and include

5 diluents of various buffer content (e.g., Tris-HCl, acetate, phosphate), pH and ionic strength, additives such as albumin or gelatin to prevent absorption to surfaces, detergents (e.g., Tween 20, Tween 80, Pluronic F68, bile acid salts). Solubilizing agents (e.g., glycerol, polyethylene glycerol), anti-oxidants (e.g., ascorbic acid, sodium metabisulfite), preservatives (e.g., thimerosal, benzyl

10 alcohol, parabens), bulking substances or tonicity modifiers (e.g., lactose, mannitol), covalent attachment of polymers such as polyethylene glycol to the protein, complexation with metal ions, or incorporation of the material into or onto particulate preparations of polymeric compounds such as polylactic acid, polyglycolic acid, hydrogels, etc, or onto liposomes, microemulsions, micelles,

15 unilamellar or multilamellar vesicles, erythrocyte ghosts, or spheroplasts. Such compositions will influence the physical state, solubility, stability, rate of in vivo release, and rate of in vivo clearance. Controlled or sustained release compositions include formulation in lipophilic depots (e.g., fatty acids, waxes, oils). Also comprehended by the invention are particulate compositions coated with

20 polymers (e.g., poloxamers or poloxamines). Other embodiments of the compositions of the invention incorporate particulate forms protective coatings, protease inhibitors or permeation enhancers for various routes of administration, including parenteral, pulmonary, nasal, oral, vaginal, rectal routes. In one embodiment the pharmaceutical composition is administered parenterally,

25 paracancerally, transmucosally, transdermally, intramuscularly, intravenously, intradermally, subcutaneously, intraperitoneally, intraventricularly, intracranially and intratumorally.

Further, as used herein "pharmaceutically acceptable carrier" or "pharmaceutical

30 carrier" are known in the art and include, but are not limited to, 0.01-0.1 M or 0.05 M phosphate buffer or 0.8 % saline. Additionally, such pharmaceutically acceptable carriers may be aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl

oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's or fixed oils. Intravenous vehicles include fluid and nutrient
5 replenishers, electrolyte replenishers such as those based on Ringer's dextrose, and the like. Preservatives and other additives may also be present, such as, for example, antimicrobials, antioxidants, collating agents, inert gases and the like.

A "analogue" is to be understood herein as a polypeptide originating from an
10 original sequence or from a portion of an original sequence and which may comprise one or more modification; for example, one or more modification in the amino acid sequence (e.g., an amino acid addition, deletion, insertion, substitution etc.), one or more modification in the backbone or side-chain of one or more
15 amino acid, or an addition of a group or another molecule to one or more amino acids (side-chains or backbone). An "analogue" is therefore understood herein as a molecule having a biological activity and chemical structure (or a portion of its structure) similar to that of a polypeptide described herein. An analog comprises a polypeptide which may have, for example, one or more amino acid insertion,
20 either at one or both of the ends of the polypeptide and/or inside the amino acid sequence of the polypeptide.

An "analogue" may have sequence similarity and/or sequence identity with that of an original sequence or a portion of an original sequence and may also have a modification of its structure as discussed herein. The degree of similarity between
25 two sequences is based upon the percentage of identities (identical amino acids) and of conservative substitution.

Similarity or identity may be compared, for example, over a region of 2, 3, 4, 5, 10, 19, 20 amino acids or more (and any number therebetween). Identity may include
30 herein, amino acids which are identical to the original peptide and which may occupy the same or similar position when compared to the original polypeptide. An analog which have, for example, 50% identity with an original polypeptide may include for example, an analog comprising 50% of the amino acid sequence of the original polypeptide and similarly with the other percentages. It is to be understood

herein that gaps may be found between the amino acids of an analogs which are identical or similar to amino acids of the original peptide. The gaps may include no amino acids, one or more amino acids which are not identical or similar to the original peptide. Biologically active analogs of the carriers (polypeptides) of the present invention are encompassed herewith.

Percent identity may be determined, for example, with an algorithm GAP, BESTFIT, or FASTA in the Wisconsin Genetics Software Package Release 7.0, using default gap weights.

For example an analogue may comprise or have 50% identity with an original amino acid sequence and a portion of the remaining amino acid which occupies a similar position may be for example a non-conservative or conservative amino acid substitution.

Therefore, analogues of the present invention comprises those which may have at least 90 % sequence similarity with an original sequence or a portion of an original sequence. An "analogue" may have, for example at least 35%, 50 %, 60%, 70%, 80%, 90% or 95% (96%, 97%, 98%, 99% and 100%) sequence similarity with an original sequence or a portion of an original sequence. Also, an "analogue" may also have, for example, at least 35%, 50 %, 60%, 70%, 80%, 90% or 95% (96%, 97%, 98%, 99% and 100%) sequence similarity to an original sequence with a combination of one or more modification in a backbone or side-chain of an amino acid, or an addition of a group or another molecule, etc. Exemplary amino acids which are intended to be similar (a conservative amino acid) to others are known in the art and includes, for example, those listed in Table 1.

Analogues of the present invention also comprises those which may have at least 35%, 50 %, 60%, 70%, 80%, 90% or 95% (96%, 97%, 98%, 99% and 100%) sequence identity with an original sequence or a portion of an original sequence. Also, an "analogue" may have, for example, 35%, 50 %, 60%, 70%, 80%, 90% or 95% (sequence) identity to an original sequence (i.e., an analogue that is at least 35%, 50 %, 60%, 70%, 80%, 90% or 95% identical to an original peptide) with a

combination of one or more modification in a backbone or side-chain of an amino acid, or an addition of a group or another molecule, etc.

5 A "fragment" is to be understood herein as a polypeptide originating from a portion of an original or parent sequence or from an analogue of said parent sequence. Fragments encompass polypeptides having truncations of one or more amino acids, wherein the truncation may originate from the amino terminus (N-terminus), carboxy terminus (C-terminus), or from the interior of the protein. A fragment may comprise the same sequence as the corresponding portion of the original
10 sequence. Biologically active fragments of the carrier (polypeptide) described herein are encompassed by the present invention.

Thus, biologically active polypeptides in the form of the original polypeptides, fragments (modified or not), analogues (modified or not), derivatives (modified or
15 not), homologues, (modified or not) of the carrier described herein are encompassed by the present invention.

Therefore, any polypeptide having a modification compared to an original polypeptide which does not destroy significantly a desired biological activity is
20 encompassed herein. It is well known in the art, that a number of modifications may be made to the polypeptides of the present invention without deleteriously affecting their biological activity. These modifications may, on the other hand, keep or increase the biological activity of the original polypeptide or may optimize one or more of the particularity (e.g. stability, bioavailability, etc.) of the
25 polypeptides of the present invention which, in some instance might be needed or desirable. Polypeptides of the present invention comprises for example, those containing amino acid sequences modified either by natural processes, such as posttranslational processing, or by chemical modification techniques which are known in the art. Modifications may occur anywhere in a polypeptide including the
30 polypeptide backbone, the amino acid side-chains and the amino- or carboxy-terminus. It will be appreciated that the same type of modification may be present in the same or varying degrees at several sites in a given polypeptide. Also, a given polypeptide may contain many types of modifications. Polypeptides may be branched as a result of ubiquitination, and they may be cyclic, with or without

- branching. Cyclic, branched and branched cyclic polypeptides may result from posttranslational natural processes or may be made by synthetic methods. Modifications comprise for example, without limitation, pegylation, acetylation, acylation, addition of acetamidomethyl (Acm) group, ADP-ribosylation, alkylation, amidation, biotinylation, carbamoylation, carboxyethylation, esterification, covalent attachment to flavin, covalent attachment to a heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of drug, covalent attachment of a marker (e.g., fluorescent, radioactive, etc.), covalent attachment of a lipid or lipid derivative, covalent attachment of phosphatidylinositol, cross-linking, cyclization, disulfide bond formation, demethylation, formation of covalent cross-links, formation of cystine, formation of pyroglutamate, formylation, gamma-carboxylation, glycosylation, GPI anchor formation, hydroxylation, iodination, methylation, myristoylation, oxidation, proteolytic processing, phosphorylation, prenylation, racemization, selenoylation, sulfation, transfer-RNA mediated addition of amino acids to proteins such as arginylation and ubiquitination, etc. It is to be understood herein that more than one modification to the polypeptides described herein are encompassed by the present invention to the extent that the biological activity is similar to the original (parent) polypeptide.
- As discussed above, polypeptide modification may comprise, for example, amino acid insertion (i.e., addition), deletion and substitution (i.e., replacement), either conservative or non-conservative (e.g., D-amino acids, desamino acids) in the polypeptide sequence where such changes do not substantially alter the overall biological activity of the polypeptide.
- Example of substitutions may be those, which are conservative (i.e., wherein a residue is replaced by another of the same general type or group) or when wanted, non-conservative (i.e., wherein a residue is replaced by an amino acid of another type). In addition, a non-naturally occurring amino acid may substitute for a naturally occurring amino acid (i.e., non-naturally occurring conservative amino acid substitution or a non-naturally occurring non-conservative amino acid substitution).

As is understood, naturally occurring amino acids may be sub-classified as acidic, basic, neutral and polar, or neutral and non-polar. Furthermore, three of the encoded amino acids are aromatic. It may be of use that encoded polypeptides differing from the determined polypeptide of the present invention contain substituted codons for amino acids, which are from the same type or group as that of the amino acid be replaced. Thus, in some cases, the basic amino acids Lys, Arg and His may be interchangeable; the acidic amino acids Asp and Glu may be interchangeable; the neutral polar amino acids Ser, Thr, Cys, Gln, and Asn may be interchangeable; the non-polar aliphatic amino acids Gly, Ala, Val, Ile, and Leu are interchangeable but because of size Gly and Ala are more closely related and Val, Ile and Leu are more closely related to each other, and the aromatic amino acids Phe, Trp and Tyr may be interchangeable.

It should be further noted that if the polypeptides are made synthetically, substitutions by amino acids, which are not naturally encoded by DNA (non-naturally occurring or unnatural amino acid) may also be made.

A non-naturally occurring amino acid is to be understood herein as an amino acid which is not naturally produced or found in a mammal. A non-naturally occurring amino acid comprises a D-amino acid, an amino acid having an acetylaminoethyl group attached to a sulfur atom of a cysteine, a pegylated amino acid, etc. The inclusion of a non-naturally occurring amino acid in a defined polypeptide sequence will therefore generate a derivative of the original polypeptide. Non-naturally occurring amino acids (residues) include also the omega amino acids of the formula $\text{NH}_2(\text{CH}_2)_n\text{COOH}$ wherein n is 2-6, neutral nonpolar amino acids, such as sarcosine, t-butyl alanine, t-butyl glycine, N-methyl isoleucine, norleucine, etc. Phenylglycine may substitute for Trp, Tyr or Phe; citrulline and methionine sulfoxide are neutral nonpolar, cysteic acid is acidic, and ornithine is basic. Proline may be substituted with hydroxyproline and retain the conformation conferring properties.

It is known in the art that analogues may be generated by substitutional mutagenesis and retain the biological activity of the polypeptides of the present invention. These analogues have at least one amino acid residue in the protein

molecule removed and a different residue inserted in its place. Examples of substitutions identified as "conservative substitutions" are shown in Table 1. If such substitutions result in a change not desired, then other type of substitutions, denominated "exemplary substitutions" in Table 1, or as further described herein in reference to amino acid classes, are introduced and the products screened.

In some cases it may be of interest to modify the biological activity of a polypeptide by amino acid substitution, insertion, or deletion. For example, modification of a polypeptide may result in an increase in the polypeptide's biological activity, may modulate its toxicity, may result in changes in bioavailability or in stability, or may modulate its immunological activity or immunological identity. Substantial modifications in function or immunological identity are accomplished by selecting substitutions that differ significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation. (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. Naturally occurring residues are divided into groups based on common side chain properties:

- (1) hydrophobic: norleucine, methionine (Met), Alanine (Ala), Valine (Val), Leucine (Leu), Isoleucine (Ile), Histidine (His), Tryptophan (Trp), Tyrosine (Tyr), Phenylalanine (Phe),
- (2) neutral hydrophilic: Cysteine (Cys), Serine (Ser), Threonine (Thr)
- (3) acidic/negatively charged: Aspartic acid (Asp), Glutamic acid (Glu)
- (4) basic: Asparagine (Asn), Glutamine (Gln), Histidine (His), Lysine (Lys), Arginine (Arg)
- (5) residues that influence chain orientation: Glycine (Gly), Proline (Pro);
- (6) aromatic: Tryptophan (Trp), Tyrosine (Tyr), Phenylalanine (Phe), Histidine (His),
- (7) polar: Ser, Thr, Asn, Gln
- (8) basic positively charged: Arg, Lys, His, and;
- (9) charged : Asp, Glu, Arg, Lys, His

Non-conservative substitutions will entail exchanging a member of one of these classes for another. A conservative substitution will entail exchanging a member of one of these groups for another member of these groups. Alternatively other conservative amino acid substitutions are listed in Table 1.

5

Table 1. amino acid substitution

Original residue	Exemplary substitution	Conservative substitution
Ala (A)	Val, Leu, Ile	Val
Arg (R)	Lys, Gln, Asn	Lys
Asn (N)	Gln, His, Lys, Arg	Gln
Asp (D)	Glu	Glu
Cys (C)	Ser	Ser
Gln (Q)	Asn	Asn
Glu (E)	Asp	Asp
Gly (G)	Pro	Pro
His (H)	Asn, Gln, Lys, Arg	Arg
Ile (I)	Leu, Val, Met, Ala, Phe, norleucine	Leu
Leu (L)	Norleucine, Ile, Val, Met, Ala, Phe	Ile
Lys (K)	Arg, Gln, Asn	Arg
Met (M)	Leu, Phe, Ile	Leu
Phe (F)	Leu, Val, Ile, Ala	Leu
Pro (P)	Gly	Gly
Ser (S)	Thr	Thr
Thr (T)	Ser	Ser
Trp (W)	Tyr	Tyr
Tyr (Y)	Trp, Phe, Thr, Ser	Phe
Val (V)	Ile, Leu, Met, Phe, Ala, norleucine	Leu

A biologically active analog may be, for example, an analogue having at least one (i.e., non-conservative or conservative) amino acid substitution in the original

- 30 -

sequence. A biologically active analog may also be for example, an analog having an insertion of one or more amino acids.

Other exemplary analogs includes for example:

- 5 - A SEQ ID NO.1 analog which may have the formula I : X_1 -SEQ ID NO.:1- X_2
- An Angiopep-1 analog which may have the formula II : X_1 -Angiopep-1- X_2 and
- An Angiopep-2 analog may have the formula III : X_1 -Angiopep-2- X_2

10 X_1 and X_2 may independently be an amino acid sequence of from between 0 to about 100 (e.g., from between 0 to about 30 to 50) amino acids. X_1 and X_2 may be derived from consecutive amino acids of aprotinin or aprotinin analogs (homologous amino acid sequence) or may be any other amino acid sequence (heterologous amino acid sequence). A compound of either formula I, II or III may also comprises an amino acid substitution, deletion or insertion within the amino

15 acid sequence of Angiopep-1, Angiopep-2 or SEQ ID NO.1. The analog however would preferably be biologically active as determined by one of the assays described herein or by any similar or equivalent assays.

A biologically active polypeptide (e.g., carrier) may be identified by using one of

20 the assays or methods described herein. For example a candidate carrier may be produced by conventional peptide synthesis, conjugated with Taxol as described herein and tested in an *in vivo* model as described herein. A biologically active carrier may be identified, for example, based on its efficacy to increase survival of an animal which has been injected with tumor cells and treated with the conjugate

25 compared to a control which has not been treated with a conjugate. Also a biologically active carrier may be identified based on its location in the parenchyma in an *in situ* cerebral perfusion assay.

It is to be understood herein, that if a "range" or "group of substances" is

30 mentioned with respect to a particular characteristic (e.g., temperature, concentration, time and the like) of the present invention, the present invention relates to and explicitly incorporates herein each and every specific member and combination of sub-ranges or sub-groups therein whatsoever. Thus, any specified range or group is to be understood as a shorthand way of referring to each and

every member of a range or group individually as well as each and every possible sub-ranges or sub-groups encompassed therein; and similarly with respect to any sub-ranges or sub-groups therein. Thus, for example,

- 5 - with respect to a length of from 10 to 18 amino acid l, is to be understood as specifically incorporating herein each and every individual length, e.g., a length of 18, 17, 15, 10, and any number therebetween etc.; Therefore, unless specifically mentioned, every range mentioned herein is to be understood as being inclusive. For example, in the expression from 5 to 10 amino acids long is to be as including 5 and 10;
- 10 - and similarly with respect to other parameters such as sequences, length, concentrations, elements, etc...

It is in particular to be understood herein that the sequences, regions, portions defined herein each include each and every individual sequences, regions, portions described thereby as well as each and every possible sub-sequences, sub-regions, sub-portions whether such sub-sequences, sub-regions, sub-portions is defined as positively including particular possibilities, as excluding particular possibilities or a combination thereof; for example an exclusionary definition for a region may read as follows: "provided that said polypeptide is no shorter than 4, 5, 6, 7, 8 or 9 amino acids. Yet a further example of a negative limitation is the following; a sequence comprising SEQ ID NO.: X with the exclusion of a polypeptide of SEQ ID NO. Y; etc. An additional example of a negative limitation is the following; provided that said polypeptide is not (does not comprise or consist of) SEQ ID NO.:Z.

25

BRIEF DESCRIPTION OF THE DRAWINGS

In drawings which illustrates exemplary embodiments of the invention,

30 Fig. 1 illustrates an example of analysis using Tricine gels;

Fig. 2 illustrates the method of attachment of the vector or carrier of the present invention to paclitaxel;

Fig. 3 illustrates the effect of treatment of glioblastoma model in Lewis rats with paclitaxel conjugated to aprotinin;

Fig. 4 illustrates the effect of treatment of glioblastoma model in nude mice with paclitaxel conjugated to AngioPep-1;

5 Fig. 5 illustrates the protocol used to conjugate aprotinin with IgG using cross-linker BS³;

Fig. 6 illustrates the protocol used to conjugate aprotinin with IgG using cross-linker sulfo-EMCS;

Fig. 7 illustrates the brain penetration for IgG-aprotinin conjugates;

10 Fig. 9 illustrates the effect of treatment of Taxol-Angiopep-2 conjugate on the survival of glioblastoma-implanted mice (athymic, nude mice) and;

Fig. 9 illustrates the structure of exemplary polypeptides of the present invention.

15

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to new molecules that can act as vectors or carriers for transporting an agent, medicine or other molecule to the brain and/or central nervous system (CNS). Agents, medicines or other molecules which are unable
20 or ineffective at crossing the blood-brain barrier by themselves, will be transported across the blood-brain barrier when attached or coupled (conjugated) to the vector or carrier. Alternatively, an agent that is able to cross the blood-brain barrier by itself may also see its transport increase when conjugate to the carrier of the present invention. Such conjugates can be in the form of a composition, such as
25 a pharmaceutical composition, for treatment of a condition or disease.

Design of Candidate Molecules as Carrier Vectors

In international publication no. WO2004/060403, the inventors have disclosed that AngioPep-1 (SEQ ID NO.:67) and aprotinin (SEQ ID NO.:98) are effective vectors

for transporting desirable molecules across the blood brain barrier. The inventors herein demonstrate that other molecules could also be used as carriers for transporting an agent across the blood brain barrier. Accordingly, peptides having similar domains as aprotinine and Angiopep-1 and a modified form of Angiopep-1 (amidated, peptide no.67) were therefore conceived as potential carrier vectors. These derived peptides resemble aprotinine and Angiopep-1 but comprise different amino acid insertions and bear different charges. Thus far, 96 peptides presented in Table 2 as well as additional peptides listed in the sequence listing were tested for their potential as carrier.

It is to be understood herein that in the following experiments, peptides have been selected based on their higher activity compared to others. Those which have not been selected for further experimentations are by no means being disclaimed and are not intended to be regarded as non-functional. These peptides show substantial activity and have utility as (biologically active) carriers and are also encompassed by the present invention.

Table 2 Design of 96 peptides from similar domain to aprotinine and Angiotensin-1 with different charges and amino acid insertions

96 PEPTIDES ORDERED AT SYNPEP (California, USA)																										
Proteins	Characteristics	#Pep	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22		
Aprot-synth Bikunin HI-30 Amyloid Kunitz-Inhib 1 Peptides	CHARGE (+6)	1	T	F	V	Y	G	G	C	R	A	K	R	N	N	N	F	K	S	A	E	K	D	E	Y	
		2	T	F	F	Q	Y	G	G	C	M	G	N	R	N	N	N	F	D	T	E	E	E	E	Y	
		3	P	F	F	Y	G	G	C	L	G	N	R	N	N	N	N	F	D	T	E	E	E	E	Y	
		4	S	T	F	Y	G	G	C	L	R	A	K	R	N	N	N	F	L	R	E	E	E	E	Y	
		5	T	F	F	F	Y	G	G	C	R	A	K	R	N	N	N	F	K	R	A	K	K	Y	Y	
		6	T	F	F	F	Y	G	G	C	R	A	K	R	N	N	N	F	K	R	A	K	K	Y	Y	
		7	T	F	F	F	Y	G	G	C	R	A	K	K	K	N	N	F	K	R	A	K	K	Y	Y	
		8	T	F	F	F	Y	G	G	C	R	A	K	K	N	N	N	F	K	R	A	K	K	Y	Y	
		9	T	F	F	Q	Y	G	G	C	R	A	K	K	R	N	N	N	F	K	R	A	K	K	Y	Y
		10	T	F	F	Q	Y	G	G	C	R	A	K	K	R	N	N	N	F	K	R	A	K	K	Y	Y
CHARGE (+5)	11	T	F	F	F	Y	G	G	C	L	G	K	K	R	N	N	F	K	R	A	K	K	Y	Y		
	12	T	F	F	F	Y	G	G	C	L	G	K	K	N	K	N	F	K	R	A	K	K	Y	Y		
	13	P	F	F	F	Y	G	G	C	R	G	K	K	K	N	N	F	K	R	A	K	K	Y	Y		
	14	T	F	F	F	Y	G	G	C	R	G	K	K	R	N	N	N	F	L	R	E	E	E	Y		
	15	P	F	F	F	Y	G	G	C	R	G	K	K	R	N	N	N	F	L	R	E	E	E	Y		
	16	T	F	F	F	Y	G	G	C	R	G	K	K	R	N	N	N	F	K	R	A	K	K	Y		
	17	P	F	F	F	Y	G	G	C	R	A	K	K	R	N	N	N	F	K	R	A	K	K	Y		
	18	T	F	F	F	Y	G	G	C	R	A	K	K	R	N	N	N	F	K	R	A	K	K	Y		
	19	T	F	F	F	Y	G	G	C	R	A	K	K	R	N	N	N	F	D	R	A	K	K	Y		
	20	T	F	F	F	Y	G	G	C	R	A	K	K	R	N	N	N	F	D	R	A	K	K	Y		
CHARGE (+4)	21	P	F	F	F	Y	G	G	C	R	A	K	K	R	N	N	N	F	D	R	A	K	K	Y		
	22	T	F	F	F	Y	G	G	C	R	A	K	K	R	N	N	N	F	L	R	E	E	E	Y		
	23	T	F	F	F	Y	G	G	C	R	A	K	K	R	N	N	N	F	L	R	E	E	E	Y		
	24	T	F	F	F	Y	G	G	C	R	A	K	K	R	N	N	N	F	L	R	E	E	E	Y		
	25	T	F	F	F	Y	G	G	S	R	G	N	R	N	N	N	F	K	T	A	K	K	Y	Y		
	19	T	F	F	F	Y	G	G	C	R	A	K	K	R	N	N	N	F	D	R	A	K	K	Y		
CHARGE (+3)	26	T	F	F	F	Y	G	G	C	L	G	N	R	N	N	N	F	K	R	A	K	K	Y	Y		
	27	T	F	F	F	Y	G	G	C	L	G	N	R	N	N	N	F	K	R	A	K	K	Y	Y		
	28	T	F	F	F	Y	G	G	C	L	R	G	N	N	N	N	F	K	S	A	K	K	Y	Y		
	29	T	F	F	F	Y	G	G	C	R	G	K	K	R	N	N	N	F	D	R	E	E	E	Y		
	30	T	F	F	F	Y	G	G	C	R	G	K	K	R	N	N	N	F	D	R	E	E	E	Y		
	31	T	F	F	F	Y	G	G	C	R	G	K	K	R	N	N	N	F	L	R	E	E	E	Y		
	32	T	F	F	F	Y	G	G	C	R	G	K	K	R	N	N	N	F	L	R	E	E	E	Y		
	33	T	F	F	F	Y	G	G	S	R	G	K	K	R	N	N	N	F	D	R	E	E	E	Y		
	CHARGE (+2)	34	T	F	F	F	Y	G	G	C	R	G	N	R	N	N	N	F	V	T	A	K	K	Y	Y	
		35	P	F	F	F	Y	G	G	C	L	G	N	R	N	N	N	F	V	T	A	K	K	Y	Y	
36		T	F	F	F	Y	G	G	C	R	G	K	K	R	N	N	N	F	L	T	A	K	K	Y		
37		S	F	F	F	Y	G	G	C	L	G	N	K	K	N	N	N	F	L	T	A	K	K	Y		
38		T	F	F	F	Y	G	G	C	M	G	N	K	N	N	N	F	V	T	E	E	E	E	Y		
39		T	F	F	F	Y	G	G	C	M	G	N	K	N	N	N	F	V	T	E	E	E	E	Y		
40		T	F	F	F	Y	G	G	S	M	G	N	K	N	N	N	F	V	T	E	E	E	E	Y		
41		P	F	F	F	Y	G	G	C	L	G	N	R	N	N	N	F	V	T	E	E	E	E	Y		
42		T	F	F	F	Y	G	G	C	L	G	N	R	N	N	N	F	V	T	E	E	E	E	Y		
43		T	F	F	F	Y	G	G	C	L	G	N	K	N	N	N	F	V	T	E	E	E	E	Y		
CHARGE (+1)	44	T	F	F	F	Y	G	G	C	R	G	N	R	N	N	N	F	L	T	A	K	K	Y	Y		
	45	T	F	F	F	Y	G	G	C	R	G	N	R	N	N	N	F	L	T	A	K	K	Y	Y		
	46	T	F	F	F	Y	G	G	C	R	L	G	N	N	N	N	F	L	T	A	K	K	Y	Y		
	47	P	F	F	F	Y	G	G	C	L	G	N	K	K	R	N	N	F	L	T	A	K	K	Y		
	48	T	F	F	F	Y	G	G	C	R	G	K	R	N	N	N	F	L	T	A	K	K	Y	Y		
	49	T	F	F	F	Y	G	G	C	R	G	K	R	N	N	N	F	L	T	A	K	K	Y	Y		
	50	P	F	F	F	Y	G	G	C	R	G	N	R	N	N	N	F	V	T	E	E	E	E	Y		
	51	S	F	F	F	Y	G	G	C	M	G	N	G	N	N	N	F	V	T	E	E	E	E	Y		
	52	P	F	F	F	Y	G	G	C	L	G	N	R	N	N	N	F	V	T	E	E	E	E	Y		
	53	T	F	F	F	Y	G	G	C	L	G	N	R	N	N	N	F	V	T	E	E	E	E	Y		
CHARGE (+0)	54	S	F	F	F	Y	G	G	C	L	G	N	R	N	N	N	F	L	R	E	E	E	E	Y		
	55	T	F	F	F	Y	G	G	S	L	G	N	R	N	N	N	F	V	T	E	E	E	E	Y		
	56	T	F	F	F	Y	G	G	C	R	G	N	R	N	N	N	F	V	T	A	K	K	Y	Y		
	57	T	F	F	F	Y	G	G	C	L	G	K	N	R	N	N	F	V	T	A	K	K	Y	Y		
	58	T	F	F	F	Y	G	G	C	L	G	K	N	R	N	N	F	V	T	A	K	K	Y	Y		
	59	T	F	F	F	Y	G	G	C	L	G	N	R	N	N	N	F	L	R	E	E	E	E	Y		
	60	T	F	F	F	Y	G	G	C	L	G	N	K	N	N	N	F	L	R	E	E	E	E	Y		
vs APROTININ M-term (1 helix α, A-term) (2 β sheets, Y-term) (1 α, 1 β)	61	P	F	F	F	Y	G	G	C	S	G	N	R	N	N	N	F	L	R	E	E	E	E	Y		
	62	P	F	F	F	Y	G	G	S	G	N	R	N	N	N	N	F	L	R	E	E	E	E	Y		
	63	M	R	P	D	F	C	L	E	P	N	Y	T	G	P	C	V	A	R	I	Y	G	Y	Y		
	64	A	R	I	I	R	Y	F	Y	N	N	A	Y	K	A	S	A	Q	C	F	Y	G	Y	Y		
	65	Y	G	R	A	K	R	N	N	N	N	Y	T	G	P	C	V	A	R	I	Y	G	Y	Y		
	66	P	D	F	C	L	E	P	P	Y	T	G	P	C	V	A	R	I	Y	G	Y	Y	Y	Y		
	67	T	F	F	F	Y	G	G	C	R	R	G	K	K	R	N	N	F	K	T	E	E	E	E	Y	
AngioPep	AngioPep-1	68	K	F	F	Y	G	G	C	R	R	G	K	K	R	N	N	F	K	T	E	E	E	E	Y	
	AngioPep1 (lysine)	69	T	F	F	Y	Y	G	G	C	R	R	G	K	K	R	N	N	F	K	T	E	E	E	Y	
	AngioPep1 (47)	70	T	F	F	F	Y	G	G	C	R	R	G	K	K	R	N	N	F	K	T	E	E	E	Y	
	cys bridge	71	C	T	F	F	Y	G	G	C	R	R	G	K	K	R	N	N	F	K	T	E	E	E	Y	
	cys-Nterminal	72	T	F	F	F	Y	G	G	C	R	R	G	K	K	R	N	N	F	K	T	E	E	E	Y	
	cys-Cterminal	73	C	T	F	F	Y	G	G	C	R	R	G	K	K	R	N	N	F	K	T	E	E	E	Y	
	cys-Nterminal	74	T	F	F	F	Y	G	G	S	R	G	K	K	R	N	N	N	F	K	T	E	E	E	Y	
	pro	75	P	F	F	F	Y	G	G	C	R	R	G	K	K	R	N	N	F	K	T	E	E	E	Y	
	charge (+3)	76	T	F	F	F	Y	G	G	C	R	R	G	K	K	R	N	N	F	K	T	E	E	E	Y	
	charge (+3)-cys	77	T	F	F	F	Y	G	G	C	R	R	G	K	K	R	N	N	F	K	T	E	E	E	Y	
	charge (+4)	78	T	F	F	F	Y	G	G	C	R	R	G	K	K	R	N	N	F	K	T	E	E	E	Y	
	charge (+4)-cys	79	T	F	F	F	Y	G	G	C	R	R	G	K	K	R	N	N	F	K	T	E	E	E	Y	
	charge (+5)	80	T	F	F	F	Y	G	G	C	R	R	G	K	K	R	N	N	F	K	T	E	E	E	Y	
	charge (+6)	81	T	F	F	F	Y	G	G	C	R	R														

Selection With *In Vitro* Model

An *in vitro* model was used for screening assay and for mechanistic studies of drug transport to the brain. This efficient *in vitro* model of the blood-brain barrier was developed by the company CELLIAL™ Technologies. Yielding reproducible results, the *in vitro* model was used for evaluating the capacity of different carriers to reach the brain. The model consists of a co-culture of bovine brain capillary endothelial cells and rat glial cells. It presents ultrastructural features characteristic of brain endothelium including tight junctions, lack of fenestration, lack of transendothelial channels, low permeability for hydrophilic molecules and a high electrical resistance. Moreover, this model has shown a good correlation coefficient between *in vitro* and *in vivo* analysis of wide range of molecules tested. To date, all the data obtained show that this BBB model closely mimics the *in vivo* situation by reproducing some of the complexities of the cellular environment that exist *in vivo*, while retaining the experimental advantages associated with tissue culture. Many studies have validated this cell co-culture as one of the most reproducible *in vitro* model of the BBB.

The *in vitro* model of BBB was established by using a co-culture of BBCECs and astrocytes. Prior to cell culture, plate inserts (Millicell-PC 3.0 µM; 30-mm diameter) were coated on the upper side with rat tail collagen. They were then set in six-well microplates containing the astrocytes and BBCECs were plated on the upper side of the filters in 2 mL of co-culture medium. This BBCEC medium was changed three times a week. Under these conditions, differentiated BBCECs formed a confluent monolayer 7 days later. Experiments were performed between 5 and 7 days after confluence was reached. The permeability coefficient for sucrose was measured to verify the endothelial permeability.

Primary cultures of mixed astrocytes were prepared from newborn rat cerebral cortex (Dehouck M.P., Meresse S., Delorme P., Fruchart J.C., Cecchelli, R. An Easier, Reproducible, and Mass-Production Method to Study the Blood-Brain Barrier *In Vitro*. *J.Neurochem*, 54, 1798-1801, 1990). Briefly, after removing the meninges, the brain tissue was forced gently through an 82 µm nylon sieve. Astrocytes were plated on six-well microplates at a concentration of 1.2×10^5 cells/mL in 2 mL of optimal culture medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum. The medium was changed twice a week.

Bovine brain capillary endothelial cells (BBCECs) were obtained from Cellial Technologies. The cells were cultured in the presence of DMEM medium supplemented with 10% (v/v) horse serum and 10% heat-inactivated calf serum, 2 mM of glutamine, 50 µg/mL of gentamycin, and 1 ng/mL of basic fibroblast growth factor, added every other day.

Originally, at a first level of selection, 96 peptides as described in Table 2 were tested as carrier with the *in vitro* model of the BBB. Each peptide was added to the upper side of the inserts covered or non-covered with endothelial cells for 90 minutes at 37°C. After the incubation, the peptides in the lower side of the chambers were resolved by electrophoresis. Electrophoresis gels were stained with Coomassie blue to visualize the peptides as illustrated with some peptides (without limitation) in Fig. 1. AngioPep-1 (either SEQ ID NO.:67 or peptide no.67 (amidated form)) is often used herein as a reference or for comparison purpose. In Fig. 1, each initial peptide applied to the upper side of the filters was loaded on electrophoresis gel (ini) as control. After 90 minutes of transcytosis, a volume of 50 µl from the basolateral side of the filters covered with endothelial cells (+) or non-covered (-) was also loaded on Tricine gels. To visualize the peptides gels were stained with Coomassie blue.

Following the first level of screening, peptides detected in the lower side of the chambers by Coomassie blue staining (5, 8, 45, 67, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 81, 82, 90 and 91) were selected for further study with the iodinated peptides. Briefly, the selected peptides were iodinated with standard procedures using iodo-beads from Sigma. Two iodo-beads were used for each protein. These beads were washed twice with 3 ml of phosphate buffer (PB) on a Whatman™ filter and resuspended in 60 µl of PB. ¹²⁵I (1 mCi) from Amersham-Pharmacia biotech was added to the bead suspension for 5 min at room temperature. The iodination for each peptide was initiated by adding 100 µg (80-100 µl) of the bead suspension. After an incubation of 10 min at room temperature, the supernatants were applied on a desalting column prepacked with 5 ml of cross-linked dextran™ from Pierce and ¹²⁵I-proteins were eluted with 10 ml of PBS. Fractions of 0.5 ml were collected and the radioactivity in 5 µl of each fraction was measured. Fractions corresponding to ¹²⁵I-proteins were pooled and dialyzed against

- 37 -

Ringer/Hepes buffer, pH 7.4. The efficiency of radiolabeling was between 0.6 - 1.0 x 10⁸ cpm/100 µg of protein.

The iodinated peptides were also investigated with the *in vitro* model of the BBB. Each peptide was added to upper side of the inserts covered or non-covered with
5 endothelial cells for 90 minutes at 37°C. After the incubation, peptides in the lower side of the chambers were TCA precipitated. Results were expressed as cpm ratios. For each [¹²⁵I]-peptide the number of cpm in the bottom chamber was divided by the total number of cpm added to filter covered with endothelial cells (+cells/initial) or uncovered (-cells/initial). The ratio between the number of [¹²⁵I]-
10 peptide found in the bottom chamber of filters covered with or without endothelial cells was also calculated (+cells/-cells). A very low -cells/initial ratio indicates that filters may interfere with the peptides (peptides 5 and 8). A high +cells/initial and +cells/-cells ratio indicate a better passage of the peptides across the brain endothelial cells. The results for the previously selected 18 peptides are shown in
15 Table 3.

Table 3**Results of the peptide screening following the second screening level**

#Peptides	Ratios		
	- cells /initial	+ cells /initial	+cells /-cells
5	0.111	0.051	0.46
8	0.086	0.039	0.46
45	0.163	0.049	0.30
67	0.403	0.158	0.39
70	0.143	0.032	0.23
71	0.072	0.027	0.37
72	0.209	0.029	0.014
73	0.056	0.017	0.30
74	0.146	0.036	0.24
75	0.207	0.087	0.42
76	0.222	0.084	0.38
77	0.224	0.063	0.28
78	0.125	0.075	0.60
79	0.194	0.078	0.40
81	0.203	0.088	0.43
82	0.120	0.043	0.36
90	0.284	0.134	0.47
91	0.406	0.158	0.30
Aprotinin	0.260	0.022	0.08

From these results, 12 peptides with +cells/-cells ratios generally higher than 0.35
 5 were selected namely; 5, 8, 67, 75, 76, 77, 78, 79, 81, 82, 90 and 91. Peptides
 #91 and #77 were also selected for further investigation because of their +cells/-
 cells ratios (>0.2).

The 12 selected peptides were then investigated by assessing their permeability
 coefficients using the *in vitro* BBB model. The effect of each selected peptide at
 10 250 nM on the BBB integrity was determined by measuring [¹⁴C] sucrose
 permeability in the BBB model on BBCEC monolayers grown on filters in the
 presence of astrocytes. To achieve this test, brain endothelial cell monolayers
 grown on inserts were transferred to 6-well plates containing 2 mL of Ringer-
 Hepes per well (basolateral compartment) for two hours at 37 °C. Ringer-Hepes
 15 solution was composed of 150 mM NaCl, 5.2 mM KCl, 2.2 mM CaCl₂, 0.2 mM
 MgCl₂, 6 mM NaHCO₃, 5 mM Hepes, 2.8 mM Hepes, pH 7.4. In each apical
 chamber, the culture medium was replaced by 1 mL Ringer-Hepes containing

the labeled [^{14}C]-sucrose. At different times, inserts were placed into another well. [^{14}C] sucrose passage was measured at 37°C, on filters without cells or with filters coated with BBCEC cells. The peptides are added at the start of the experiment at time zero. The results were plotted as the sucrose clearance (μl) as a function of time (min).

$$\text{Clearance } (\mu\text{l}) = \frac{[\text{C}]_{\text{A}} \times \text{VA}}{[\text{C}]_{\text{L}}}$$

[C]_A = Abluminal tracer concentration
 VA = Volume of abluminal chamber
 [C]_L = Luminal tracer concentration

The slope of the linear variation ($\mu\text{l}/\text{min}$) is the sucrose permeability coefficient for the filter without cells (P_{sf}) and one with coated with BBCEC cells (P_{St}) in the presence of the peptide.

The permeability coefficient (P_e) was calculated as:

$$1/P_e = (1/P_{\text{St}} - 1/P_{\text{sf}}) / \text{filter area } (4.2 \text{ cm}^2)$$

The peptides with highest P_e were selected: 67, 76, 90, 91, 5, 79, 8, and 78.

The *in situ* cerebral perfusion (in mice) was used as the fourth level of selection to select the best peptides. This procedure also distinguishes between compounds remaining in the brain vascular compartment from those having crossed the abluminal endothelial membrane to enter the brain parenchyma. Indeed, the technique of post-perfusion capillary depletion allows to measure whether the molecule really crosses the endothelium to enter the brain parenchyma. Using this technique it is demonstrated herein that specific peptides tend to accumulate in the brain parenchyma fraction (see Table 4).

Table 4

#Peptides		Volume of distribution (perfusion 5min)			
		Homogenate	Capillaries		Parenchyma
		(ml/100g)	(ml/100g)	%	(ml/100g) %
	5	312	217	73	95 27
5	8	250	204	82	46 18
	25	1141	1082	95	60 5
	67	38	13	34	25 65
	76	40	16	40	24 60
	78	198	181	90	16 10
	79	70	52	74	18 26
	90	87	76	88	11 12
10	91	47	24	59	23 41

Four peptides, namely 5, 67, 76 and 91, showed the highest levels of distribution in the parenchyma with a volume higher than 20 ml/100g and which represents at least 25% of the volume found for the total brain (homogenate), thus showing the highest potential as carrier for use as transport vectors. Peptide 79 was eliminated because of its lower volume of distribution in the brain parenchyma (18 ml/100g). Peptide 67 represents the amidated form of AngioPep-1 described in the previous application that the inventors filed. Amidation of a peptide affect the overall charge of the peptide. As is apparent in Tables 2 and 3, two peptides having a different charge do not have necessary the same activity.

The vector or carrier of the present invention may thus be used in a method for transporting an agent across the blood-brain barrier comprises administering to an individual an agent that comprises an active ingredient or a pharmaceutical agent attached to a carrier, such as aprotinin or a functional derivative thereof (i.e., an aprotinin analog, an aprotinin fragment, an aprotin derivative, an analogue of an aprotinin fragment).

The carrier and conjugate may be administered intra-arterially, intra-nasally, intra-peritoneally, intravenously, intramuscularly, sub-cutaneously, transdermally or *per os* to the patient. The agent may be, for example, an anti-angiogenic compound. The agent may have a maximum weight of 160,000 Daltons. As discussed
5 herein, the agent may be a marker or a drug such as a small molecule drug, a protein, a peptide or an enzyme. The drug may be adapted to treat, for example, a neurological disease or a central nervous system disorder of a patient. The drug may be a cytotoxic drug and the marker may be a detectable label such as a radioactive label, a green fluorescent protein, a histag protein or β -galactosidase.
10 The agent may be delivered, for example, into the central nervous system of a patient.

According to another embodiment, the uses, methods, compounds, agents, drugs or medicaments therein mentioned may not alter the integrity of the blood-brain barrier of the patient.

15 According to a further embodiment of the present invention the peptide may be selected from the group consisting of aprotinin, an aprotinin fragment (SEQ ID NO.:1) and any one of the peptides defined in SEQ ID NO.:1 to 97, 99, 100 or 101.

For example, peptides 5, 76, 91, 97 and 97 as well as peptide 67 may be used in
20 the present invention by linking them to an agent or a compound for transporting the agent or compound across the blood-brain barrier of a patient. The agent or compound may be adapted to treat a neurological disease or to treat a central nervous system disorder.

The carrier of the present invention, such as for example, peptides 5, 76, 91 and
25 97 as well as peptide 67 may be linked to or labelled with a detectable label such as a radioimaging agent, such as those emitting radiation, for detection of a disease or condition, for example by the use of a radioimaging agent-antibody-carrier conjugate, wherein the antibody binds to a disease or condition-specific antigen. Other binding molecules besides antibodies and which are known and
30 used in the art are also contemplated by the present invention. Alternatively, the carrier or functional derivative thereof of the present invention or mixtures thereof

may be linked to a therapeutic agent, to treat a disease or condition, or may be linked to or labelled with mixtures thereof. Treatment may be effected by administering a carrier-agent conjugate of the present invention to an individual under conditions which allow transport of the agent across the blood-brain barrier.

5 A therapeutic agent as used herein may be a drug, a medicine, an agent emitting radiation, a cellular toxin (for example, a chemotherapeutic agent) and/or biologically active fragment thereof, and/or mixtures thereof to allow cell killing or it may be an agent to treat, cure, alleviate, improve, diminish or inhibit a disease or condition in an individual treated. A therapeutic agent may be a synthetic product
10 or a product of fungal, bacterial or other microorganism, such as mycoplasma, viral etc., animal, such as reptile, or plant origin. A therapeutic agent and/or biologically active fragment thereof may be an enzymatically active agent and/or fragment thereof, or may act by inhibiting or blocking an important and/or essential cellular pathway or by competing with an important and/or essential naturally
15 occurring cellular component.

Examples of radioimaging agents emitting radiation (detectable radio-labels) that may be suitable are exemplified by indium-111, technitium-99, or low dose iodine-131.

Detectable labels, or markers, for use in the present invention may be a
20 radiolabel, a fluorescent label, a nuclear magnetic resonance active label, a luminescent label, a chromophore label, a positron emitting isotope for PET scanner, chemiluminescence label, or an enzymatic label. Fluorescent labels include but are not limited to, green fluorescent protein (GFP), fluorescein, and rhodamine. Chemiluminescence labels include but are not limited to, luciferase
25 and β -galactosidase. Enzymatic labels include but are not limited to peroxidase and phosphatase. A histag may also be a detectable label.

It is contemplated that an agent may be releasable from the carrier after transport across the blood-brain barrier, for example by enzymatic cleavage or breakage of a chemical bond between the carrier and the agent. The release agent may then
30 function in its intended capacity in the absence of the carrier.

The present invention will be more readily understood by referring to the following examples which are given to illustrate the invention rather than to limit its scope. The following examples have been given with aprotinin. However, it has been demonstrated herein the molecules of the present invention share common properties with aprotinin with respect to their potential as carrier for transporting an agent across the blood brain barrier. These examples thus apply to the molecules of the present invention.

EXAMPLE I

Strategies for drug conjugation (paclitaxel)

For conjugation, paclitaxel (TAXOL™) has 2 strategic positions (position C2' and C7). Fig. 2 illustrates the method of attachment of the vector or carrier of the present invention to paclitaxel. Briefly, paclitaxel is reacted with anhydride succinic pyridine for 3 hours at room temperature to attach a succinyl group in position 2'. Such 2'-succinyl paclitaxel has a cleavable ester bond in position 2' which upon cleavage can simply release succinic acid. This cleavable ester bond can be further used for various modifications with linkers, if desired. The resulting 2'-O-succinyl-paclitaxel is then reacted with EDC/NHS in DMSO for 9 hours at room temperature, followed by the addition of the carrier or vector in Ringer/DMSO for an additional reaction time of 4 hours at room temperature. The reaction of conjugation depicted in Fig. 2 is monitored by HPLC. Each intermediate, such as paclitaxel, 2'-O-succinyl-paclitaxel and 2'-O-NHS-succinyl-paclitaxel, is purified and validated using different approaches such as HPLC, thin liquid chromatography, NMR (¹³C or ¹H exchange), melting point, mass spectrometry. The final conjugate is analyzed by mass spectrometry and SDS-polyacrylamide gel electrophoresis. This allows determining the number of paclitaxel molecules conjugated on each vector.

Transcytosis capacity of Aprotinin-Paclitaxel conjugate was determined and is reported below in Table 5.

Table 5**Determination of aprotinin-Taxol conjugate transcytosis capacity across the BBB**

	Transcytosis (Pe 10 ⁻³ cm/min)	Sucrose Integrity (Pe 10 ⁻³ cm/min)
Control		
Aprotinin	0.2	0.28
Aprotinin-Taxol	0.21	0.24
		0.22

- **Conjugation does not affect the aprotinin capacity to cross the barrier**
- **The integrity of the barrier is also maintained**

As seen in Table 5, conjugation of paclitaxel to aprotinin still was able to cross the *in vitro* model of the blood brain barrier without affecting the sucrose integrity, thus proving that the molecules (also referred herein as vectors or carriers) of the present invention still retain their activity when conjugated to a large chemical entity such as paclitaxel.

Survival study in the rat brain tumor model was then conducted to verify whether the paclitaxel that was conjugated is still active *in vivo*. For the rat brain tumor model, rats received an intra-cerebral implantation of 50 000 CNS-1 glioma cells. Three (3) days after, animals received treatment with vehicle (aprotinin), Paclitaxel (5mg/kg) or Paclitaxel-Aprotinin (5mg/kg) by intravenous injection. Treatment was then administered every week until animal was sacrificed (see Fig. 3). Rats were monitored every day for clinical symptoms and weight loss. According to the protocol of good animal practice, animals were sacrificed when a weight loss was observed for 3 consecutive days or before if the weight loss was more than 20% of the animal initial weight.

- 45 -

Using the same experimental protocol, paclitaxel when injected alone at the maximal tolerated dose (54mg/kg) was unable to increase mouse survival (Laccabue et al., 2001 Cancer, 92 (12): 3085-92).

Survival study was also conducted in mice implanted with a human brain tumor xenograft. For the mice brain tumor model, mice received an intra-cerebral implantation of 500 000 human U87 glioma cells. 3 days after implantation animals received treatment with Paclitaxel-Angiopep1 (5mg/kg) or vehicle by intravenous injection. Treatment was then administered every week until animal was sacrificed. Mice were monitored every day for clinical symptoms and weight loss. According to the protocol of good animal practice, animals were sacrificed when a weight loss was observed for 3 consecutive days or before if the weight loss was more than 20% of the animal initial weight. It was now observed that the medium survival for the control group was 19 ± 2 days. For the statistical analysis a 20% increase in survival was considered significant. As can be seen in Fig. 4, the conjugate Paclitaxel-AngioPep-1 retained its activity, having a statistically significant effect. The survival time of the paclitaxel-angioPep1 treated animals is significantly extended when compared to control group ($p < 0.05$, $n=8$).

Results obtained in the two survival studies indicate that the conjugation of paclitaxel with the vector of the present invention increases the animal survival.

20

EXAMPLE II

Strategies for antibodies conjugation

Since proteins generally have several amino groups available for conjugation, amine coupling using sulfo-NHS/EDC activation is be used to cross-link therapeutic antibodies with the vectors (carriers) of the present invention. This approach was chosen because it is a fast, simple and reproducible coupling technique, because the resulting conjugate is stable while still retaining the biological activity of the antibody and it has a high conjugation capacity that can be reliably controlled and a low non-specific interaction during the coupling procedures.

30

Antibodies or antibody fragments (Fab and Fab'₂) have been conjugated with the vector of the present invention to increase their delivery to the brain. Various conjugation approaches have been used to first conjugate IgGs with aprotinin, having proven that the carriers of the present invention behave exactly as aprotinin.

Different cross-linkers, such as BS³ [Bis(sulfosuccinimidyl)suberate], NHS/EDC (N-hydroxysuccinimide and N-ethyl-N'-(dimethylaminopropyl)carbodiimide or Sulfo-EMCS ([N-e-Maleimidocaproic acid]hydrazide) have been tested for the conjugation of IgG. BS³ is a Homobifunctional N-hydroxysuccinimide ester that targets accessible primary amines. NHS/EDC creates a conjugation of primary amine groups with carboxyl groups. Sulfo-EMCS are heterobifunctional reactive groups (maleimide and NHS-ester) that are reactive toward: sulfhydryl and amino groups.

Conjugation of IgG with aprotinin using the cross-linker BS³ (Fig. 5) or sulfo-EMCS (Fig. 6) was first assessed.

Transport of IgG or IgG-conjugates across the BBB was then tested. The uptake of [¹²⁵I]-IgG to the luminal side of mouse brain capillaries was measured using the *in situ* brain perfusion method adapted in the inventor's laboratory for the study of drug uptake in the mouse brain (Dagenais et al., 2000, J. Cereb. Blood Flow Metab. 20(2):381-386). The BBB transport constants were determined as previously described by Smith (1996, Pharm. Biotechnol. 8:285-307). IgG uptake was expressed as the volume of distribution (Vd) from the following equation:

$$Vd = Q \cdot br / C \cdot pf$$

where Q*br is the calculated quantity of [¹²⁵I]-IgG or [¹²⁵I]-IgG-aprotinin conjugate per gram of right brain hemisphere and C*pf is the labelled tracer concentration measured in the perfusate.

The results of this experiment indicate that there is higher brain uptake for [¹²⁵I]-IgG-aprotinin conjugate than that of unconjugated [¹²⁵I]-IgG (see Fig. 7).

The conjugation of IgGs with aprotinin increases their accumulation in the brain parenchyma *in vivo*.

EXAMPLE III

Effect of Taxol-Angiopep-2 conjugate on mice survival

This study with Taxol-Angiopep-2 (herein referred to peptide no. 97 (**angiopep2 is not amidated**)) was conducted to determine whether conjugation of Taxol to Angiopep-2 could increase mice survival. The structure of Angiopep-2 is illustrated in SEQ ID NO.:97. For this experiment, mice received an intra-cerebral implantation of 500 000 human U87 glioma cells. After 3 days following implantation, animals were treated with the vehicle (DMSO/Ringer-Hepes 80:20 v/v (i.e., control)) or Taxol-Angiopep-2 conjugate (3:1, i.e., ratio of 3 Taxol molecules for each peptide; Txlan2 (5 mg/kg)) by tail vein injections (Fig. 8). Mice were monitored every day for clinical symptoms and weight loss. Treatments were administered until animals were sacrificed. As shown in Table 6, we observed that the median survival was 18 days for the control group whereas the median survival for mice receiving the Taxol-Angiopep-2 conjugate was 21 days (Fig. 8). Survival curve obtained for mice treated with Taxol-Angiopep-2 conjugate (in red) indicates that the median survival was significantly increased by 17% (Fig. 8). The statistical analysis presented also in Table 6 indicates that administration of Taxol-Angiopep-2 conjugate significantly increased survival by 17% (p values = 0.048).

Table 6. Results summary of the survival study

a. Median survival	Days	Increased (%)	Mice (n)
Control	18.0	-	7
Txlan2 conjugate	21.0	+17	7
b. Statistical analysis	(p values)		Stat. differences
Control vs Txlan2 conjugate	p = 0.048		Yes

- 48 -

The content of each publication, patent and patent application mentioned in the present application is incorporated herein by reference.

- 5 Although the present invention has been described in details herein and illustrated in the accompanying drawings, it is to be understood that the invention is not limited to the embodiments described herein and that various changes and modifications may be effected without departing from the scope or spirit of the present invention.

10

- While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such
15 departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.

20

SEQUENCES

SEQ ID

NO. :

```

1 T F V Y G G C R A K R N N F K S A E D
2 T F Q Y G G C M G N G N N F V T E K E
3 P F F Y G G C G G N R N N F D T E E Y
4 S F Y Y G G C L G N K N N Y L R E E E
5 T F F Y G G C R A K R N N F K R A K Y

```

Peptide no. 5 comprises the amino acid sequence defined in SEQ ID NO.:5 and is amidated at its N-terminus (see for example Fig. 9)

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6 T F F Y G G C R G K R N N F K R A K Y
7 T F F Y G G C R A K K N N Y K R A K Y
8 T F F Y G G C R G K K N N F K R A K Y
9 T F Q Y G G C R A K R N N F K R A K Y
10 T F Q Y G G C R G K K N N F K R A K Y
11 T F F Y G G C L G K R N N F K R A K Y
12 T F F Y G G S L G K R N N F K R A K Y
13 P F F Y G G C G G K K N N F K R A K Y
14 T F F Y G G C R G K G N N Y K R A K Y
15 P F F Y G G C R G K R N N F L R A K Y
16 T F F Y G G C R G K R N N F K R E K Y
17 P F F Y G G C R A K K N N F K R A K E
18 T F F Y G G C R G K R N N F K R A K D
19 T F F Y G G C R A K R N N F D R A K Y
20 T F F Y G G C R G K K N N F K R A E Y
21 P F F Y G G C G A N R N N F K R A K Y
22 T F F Y G G C G G K K N N F K T A K Y
23 T F F Y G G C R G N R N N F L R A K Y
24 T F F Y G G C R G N R N N F K T A K Y
25 T F F Y G G S R G N R N N F K T A K Y
26 T F F Y G G C L G N G N N F K R A K Y
27 T F F Y G G C L G N R N N F L R A K Y

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- 50 -

28 T F F Y G G C L G N R N N F K T A K Y
29 T F F Y G G C R G N G N N F K S A K Y
30 T F F Y G G C R G K K N N F D R E K Y
31 T F F Y G G C R G K R N N F L R E K E
32 T F F Y G G C R G K G N N F D R A K Y
33 T F F Y G G S R G K G N N F D R A K Y
34 T F F Y G G C R G N G N N F V T A K Y
35 P F F Y G G C G G K G N N Y V T A K Y
36 T F F Y G G C L G K G N N F L T A K Y
37 S F F Y G G C L G N K N N F L T A K Y
38 T F F Y G G C G G N K N N F V R E K Y
39 T F F Y G G C M G N K N N F V R E K Y
40 T F F Y G G S M G N K N N F V R E K Y
41 P F F Y G G C L G N R N N Y V R E K Y
42 T F F Y G G C L G N R N N F V R E K Y
43 T F F Y G G C L G N K N N Y V R E K Y

44 T F F Y G G C G G N G N N F L T A K Y
45 T F F Y G G C R G N R N N F L T A E Y
46 T F F Y G G C R G N G N N F K S A E Y
47 P F F Y G G C L G N K N N F K T A E Y
48 T F F Y G G C R G N R N N F K T E E Y
49 T F F Y G G C R G K R N N F K T E E D
50 P F F Y G G C G G N G N N F V R E K Y
51 S F F Y G G C M G N G N N F V R E K Y
52 P F F Y G G C G G N G N N F L R E K Y
53 T F F Y G G C L G N G N N F V R E K Y
54 S F F Y G G C L G N G N N Y L R E K Y
55 T F F Y G G S L G N G N N F V R E K Y
56 T F F Y G G C R G N G N N F V T A E Y
57 T F F Y G G C L G K G N N F V S A E Y
58 T F F Y G G C L G N R N N F D R A E Y
59 T F F Y G G C L G N R N N F L R E E Y
60 T F F Y G G C L G N K N N Y L R E E Y
61 P F F Y G G C G G N R N N Y L R E E Y

- 51 -

62 P F F Y G G S G G N R N N Y L R E E Y
63 M R P D F C L E P P Y T G P C V A R I
64 A R I I R Y F Y N A K A G L C Q T F V Y G
65 Y G G C R A K R N N Y K S A E D C M R T C G
66 P D F C L E P P Y T G P C V A R I I R Y F Y
67 T F F Y G G C R G K R N N F K T E E Y

The peptide no. 67 comprises the amino acid sequence defined in SEQ ID NO.:67 and is amidated at its N-terminus (see for example Fig. 9)

68 K F F Y G G C R G K R N N F K T E E Y
69 T F Y Y G G C R G K R N N Y K T E E Y
70 T F F Y G G S R G K R N N F K T E E Y
71 C T F F Y G C C R G K R N N F K T E E Y
72 T F F Y G G C R G K R N N F K T E E Y C
73 C T F F Y G S C R G K R N N F K T E E Y
74 T F F Y G G S R G K R N N F K T E E Y C
75 P F F Y G G C R G K R N N F K T E E Y
76 T F F Y G G C R G K R N N F K T K E Y

The peptide no. 76 comprises the amino acid sequence defined in SEQ ID NO.:76 and is amidated at its N-terminus (see for example Fig. 9).

77 T F F Y G G K R G K R N N F K T E E Y
78 T F F Y G G C R G K R N N F K T K R Y
79 T F F Y G G K R G K R N N F K T A E Y
80 T F F Y G G K R G K R N N F K T A G Y
81 T F F Y G G K R G K R N N F K R E K Y
81 T F F Y G G C G G N G N N F L T A K Y
82 T F F Y G G C R G N R N N F L T A E Y
83 T F F Y G G C R G N G N N F K S A E Y
84 P F F Y G G C L G N K N N F K T A E Y
85 T F F Y G G C R G N R N N F K T E E Y
86 T F F Y G G C R G K R N N F K T E E D
87 P F F Y G G C G G N G N N F V R E K Y

- 52 -

88 R F K Y G G C L G N M N N F E T L E E
 89 R F K Y G G C L G N K N N F L R L K Y
 91 R F K Y G G C L G N K N N Y L R L K Y

Peptide no. 91 comprises the amino acid sequence defined in SEQ ID NO.:91 and is amidated at its N-terminus (see for example Fig. 9).

92 K T K R K R K K Q R V K I A Y E E I F K N Y
 93 K T K R K R K K Q R V K I A Y
 94 R G G R L S Y S R R F S T S T G R
 95 R R L S Y S R R R F
 96 R Q I K I W F Q N R R M K W K K
 97 T F F Y G G S R G K R N N F K T E E Y
 98 M R P D F C L E P P Y T G P C V A R I
 I R Y F Y N A K A G L C Q T F V Y G G
 C R A K R N N F K S A E D C M R T C G G A

99 T F F Y G G C R G K R N N F K T K E Y
 100 R F K Y G G C L G N K N N Y L R L K Y
 101 T F F Y G G C R A K R N N F K R A K Y
 102 N A K A G L C Q T F V Y G G C L A K R N N F
 E S A E D C M R T C G G A

103 Y G G C R A K R N N F K S A E D C M R T C G
 G A

104 G L C Q T F V Y G G C R A K R N N F K S A E
 105 L C Q T F V Y G G C E A K R N N F K S A

SEQ ID NO.: 106

5 atgagaccag atttctgcct cgagccgccg tacactgggc cctgcaaagc tcgtatcatc
 cgttacttct acaatgcaaa ggcaggcctg tgtcagacct tcgtatacgg cggctgcaga
 gctaagcgta acaacttcaa atccgcggaa gactgcatgc gtacttgccg tgggtgcttag

WHAT IS CLAIMED IS:

1. A biologically active polypeptide able to cross a cell layer mimicking a mammalian blood brain barrier in an *in vitro* assay, said polypeptide being selected from the group of;
 - a) an aprotinin fragment comprising the amino acid sequence defined in SEQ ID NO.:1,
 - b) an aprotinin fragment consisting of SEQ ID NO.:1,
 - c) a biologically active analogue of SEQ ID NO.:1,
 - d) a biologically active fragment of SEQ ID NO.:1, and;
 - e) a biologically active fragment of a SEQ ID NO.:1 analogue.
2. A biologically active polypeptide able to cross a cell layer mimicking a mammalian blood brain barrier in an *in vitro* assay, said polypeptide being selected from the group of;
 - an aprotinin fragment of from 19 to 50 amino acid long, which may comprise SEQ ID NO.:1,
 - an aprotinin fragment consisting of SEQ ID NO.:1,
 - a biologically active analogue of SEQ ID NO.:1 of from about 19 to 50 amino acids long, provided that said analogue does not comprise SEQ ID NO.: 102, 103, 104 or 105 and provided that when said analogue consists of SEQ ID NO.:67 said analogue is amidated,
 - a biologically active fragment of SEQ ID NO.:1 of from 10 to 18 amino acids, and;
 - a biologically active fragment of a SEQ ID NO.:1 analogue of from about 10 to 18 amino acids.
3. The polypeptide of claim 1 or 2, wherein said biologically active analogue of SEQ ID NO.:1 is selected from the group consisting of;
 - a) a SEQ ID NO.:1 analogue comprising at least 35 % identity with the amino acid sequence of SEQ ID NO.:1,
 - b) a SEQ ID NO.:1 analogue comprising at least 40 % identity with the amino acid sequence of SEQ ID NO.:1,

- c) a SEQ ID NO.:1 analogue comprising at least 50 % identity with the amino acid sequence of SEQ ID NO.:1,
 - d) a SEQ ID NO.:1 analogue comprising at least 60 % identity with the amino acid sequence of SEQ ID NO.:1,
 - e) a SEQ ID NO.:1 analogue comprising at least 70 % identity with the amino acid sequence of SEQ ID NO.:1,
 - f) a SEQ ID NO.:1 analogue comprising at least 80 % identity with the amino acid sequence of SEQ ID NO.:1,
 - g) a SEQ ID NO.:1 analogue comprising at least 90 % identity with the amino acid sequence of SEQ ID NO.:1 and;
 - h) a SEQ ID NO.:1 analogue comprising at least 95 % identity with the amino acid sequence of SEQ ID NO.:1.
4. The polypeptide of claim 1, wherein said biologically active analogue of SEQ ID NO.:1 comprises an amino acid sequence selected from the group consisting of an amino acid sequence defined in SEQ ID NO.:2 to SEQ ID NO.: 62, SEQ ID NO.: 68 to SEQ ID NO.: 93 and SEQ ID NO.:97.
5. The polypeptide of claim 1, wherein said biologically active analogue of SEQ ID NO.:1 comprises the amino acid sequence defined in SEQ ID NO.:67.
6. The polypeptide of claim 5, wherein said polypeptide is amidated.
7. The polypeptide of claim 1, wherein said biologically active analogue of SEQ ID NO.:1 comprises the amino acid sequence defined in SEQ ID NO.:99, 100 or 101.
8. The polypeptide of any one of claims 1 to 4 or 7, wherein said amino acid sequence is amidated.
9. The polypeptide of claim 1, wherein said aprotinin fragment is from 10 to 50 amino acids in length.

10. The polypeptide of claim 10, wherein said aprotinin fragment is from 10 to 50 amino acids in length.
11. The polypeptide of any one of claims 4 to 7, wherein said amino acid sequence comprises of from between 1 to 12 amino acid substitutions.
12. The polypeptide of any one of claims 4 to 7, wherein said amino acid sequence comprises of from between 1 to 10 amino acid substitutions.
13. The polypeptide of any one of claims 4 to 7, wherein said amino acid sequence comprises of from between 1 to 5 amino acid substitutions.
14. The polypeptide of claim 11, wherein said amino acid substitution is non-conservative.
15. The polypeptide of claim 11, wherein said amino acid substitution is a conservative amino acid substitution.
16. The polypeptide of claim 3, wherein at least one amino acid of said analogue which is non-identical to a correspondingly located amino acid of SEQ ID NO.:1 is a conservative amino acid substitution.
17. The polypeptide of claim 1, wherein said biologically active fragment of SEQ ID NO.:1 is from 10 to 18 amino acids in length.
18. The polypeptide of claim 1, wherein said biologically active fragment of SEQ ID NO.:1 analog is from 10 to 18 amino acids in length.
19. The polypeptide of claim 17, wherein said biologically active fragment of SEQ ID NO.:1 comprises at least 10 amino acids of SEQ ID NO.1.
20. The polypeptide of claim 17, wherein said biologically active fragment of a SEQ ID NO.:1 analogue comprises at least 10 amino acids of said SEQ ID NO.:1 analogue.

21. A conjugate comprising;
- a) a carrier selected from the group consisting of the polypeptide of any one of claims 1 to 20 and;
 - b) an agent selected from the group consisting of a drug, a detectable label, a protein, protein-based compound and a polypeptide.
22. A conjugate comprising;
- a) a carrier selected from the group consisting of the polypeptide of any one of claims 1 to 20, provided that when said carrier consists of SEQ ID NO.:67 said carrier is amidated, and;
 - b) an agent selected from the group consisting of a drug, a detectable label, a protein, protein-based compound and a polypeptide.
23. The conjugate of claim 21 or 22, wherein said detectable label is a radioimaging agent.
24. The conjugate of claim 21 or 22, wherein said protein-based compound is an antibody or an antibody fragment thereof.
25. The conjugate of claim 21 or 22, wherein said agent is small molecule drug.
26. The conjugate of claim 25, wherein said small molecule drug is an anticancer drug.
27. The conjugate of claim 26, wherein said anticancer drug is selected from the group consisting of paclitaxel, vinblastine, vincristine, etoposide, doxorubicin, cyclophosphamide, taxotere, melphalan, chlorambucil and combination thereof.
28. The use of a carrier selected from the group consisting of the polypeptide of any one of claims 1 to 20 or the conjugate of any one of claims 21 to 27,

for transporting a agent across a blood brain barrier of a mammal in need thereof.

29. The use of a carrier selected from the group consisting of the polypeptide of any one of claims 1 to 20 or the conjugate of any one of claims 21 to 27, for transporting a agent across a blood brain barrier of a mammal in need thereof, provided that when said carrier consist in SEQ ID NO.:67, said carrier is amidated.
30. The use as defined in claim 28 or 29, wherein said agent is a detectable label.
31. The use as defined in claim 28 or 29, wherein said detectable label is a radioimaging agent.
32. The use as defined in claim 28 or 29, wherein said agent is a protein-based compound.
33. The use as defined in claim 32, wherein said protein-based compound is an antibody or an antibody fragment thereof.
34. The use as defined in claim 28 or 29, wherein said agent is small molecule drug.
35. The use as defined in claim 34, wherein said small molecule drug is an anticancer drug.
36. The use as defined in claim 35, wherein said anticancer drug is selected from the group consisting of paclitaxel, vinblastine, vincristine, etoposide, doxorubicin, cyclophosphamide, taxotere, melphalan, chlorambucil and combination thereof.
37. The use of a carrier selected from the group consisting of the polypeptide of any one of claims 1 to 20 or the conjugate of any one of claims 21 to 27

in the manufacture of a medicament for treating a brain or neurological disease or for the diagnostic of a brain or neurological disease.

38. The use of a carrier selected from the group consisting of the polypeptide of any one of claims 1 to 20 or the conjugate of any one of claims 21 to 27 in the manufacture of a medicament for treating a brain or neurological disease or for the diagnostic of a brain or neurological disease, provided that when said carrier consist of SEQ ID NO.:67, said carrier is amidated.
39. The use as defined in claim 37 or 38, wherein said neurological disease is selected from the group consisting of a brain tumor, a brain metastasis, schizophrenia, epilepsy, Alzheimer's disease, Parkinson's disease, Huntington's disease, stroke and blood-brain barrier related malfunctions.
40. The use as defined in claim 39, wherein said blood-brain barrier related malfunction disease is obesity.
41. A method for treating a patient having a neurological disease comprising administering a carrier selected from the group consisting of the polypeptide of any one of claims 1 to 20 or the conjugate of any one of claims 21 to 27.
42. A method for treating a patient having a neurological disease comprising administering a carrier selected from the group consisting of the polypeptide of any one of claims 1 to 20 or the conjugate of any one of claims 21 to 27, provided that when said carrier consist in SEQ ID NO.:67, said carrier is amidated.
43. A method for diagnosing a neurological disease in a patient in need thereof comprising administering a carrier selected from the group consisting of the polypeptide of any one of claims 1 to 20 or the conjugate of any one of claims 21 to 27.

44. A method for diagnosing a neurological disease in a patient in need thereof comprising administering a carrier selected from the group consisting of the polypeptide of any one of claims 1 to 20 or the conjugate of any one of claims 21 to 27, provided that when said carrier consist in SEQ ID NO.:67, said carrier is amidated.
45. The use of a carrier selected from the group consisting of the polypeptide of any one of claims 1 to 20 or the conjugate of any one of claims 21 to 27 for treating a mammal having a neurological disease or for the diagnosis of a neurological disease in a mammal in need thereof.
46. The use of a carrier selected from the group consisting of the polypeptide of any one of claims 1 to 20 or the conjugate of any one of claims 21 to 27 for treating a mammal having a neurological disease or for the diagnosis of a neurological disease in a mammal in need thereof, provided that when said carrier consist in SEQ ID NO.:67, said carrier is amidated.
47. The use as defined in claim 45 or 46, wherein said neurological disease is selected from the group consisting of a brain tumor, a brain metastasis, schizophrenia, epilepsy, Alzheimer's disease, Parkinson's disease, Huntington's disease, stroke and blood-brain barrier related malfunctions.
48. The use as defined in claim 47, wherein said blood-brain barrier related malfunction disease is obesity.
49. A pharmaceutical composition comprising
- a) a carrier selected from the group consisting of the polypeptide of any one of claims 1 to 20 or the conjugate of any one of claims 21 to 27, and;
 - b) a pharmaceutically acceptable carrier.
50. A pharmaceutical composition comprising
- a) a carrier selected from the group consisting of the polypeptide of any one of claims 1 to 20 or the conjugate of any one of claims 21

- to 27 provided that when said carrier consist in SEQ ID NO.:67,
said carrier is amidated and;
b) a pharmaceutically acceptable carrier.

51. The pharmaceutical composition of claim 49 or 50, wherein said pharmaceutical composition is used for the treatment of a neurological disease.
52. The pharmaceutical composition of claim 49 or 50, wherein said pharmaceutical composition is used for the diagnosis of a neurological disease.
53. A nucleotide sequence encoding any one of the polypeptide of claims 1 to 4 or 7.
54. The nucleotide sequence of claim 43, wherein said sequence is composed of a nucleotide selected from the group consisting of a ribonucleotide, a deoxyribonucleotide and nucleotide analogs thereof.

1 / 9

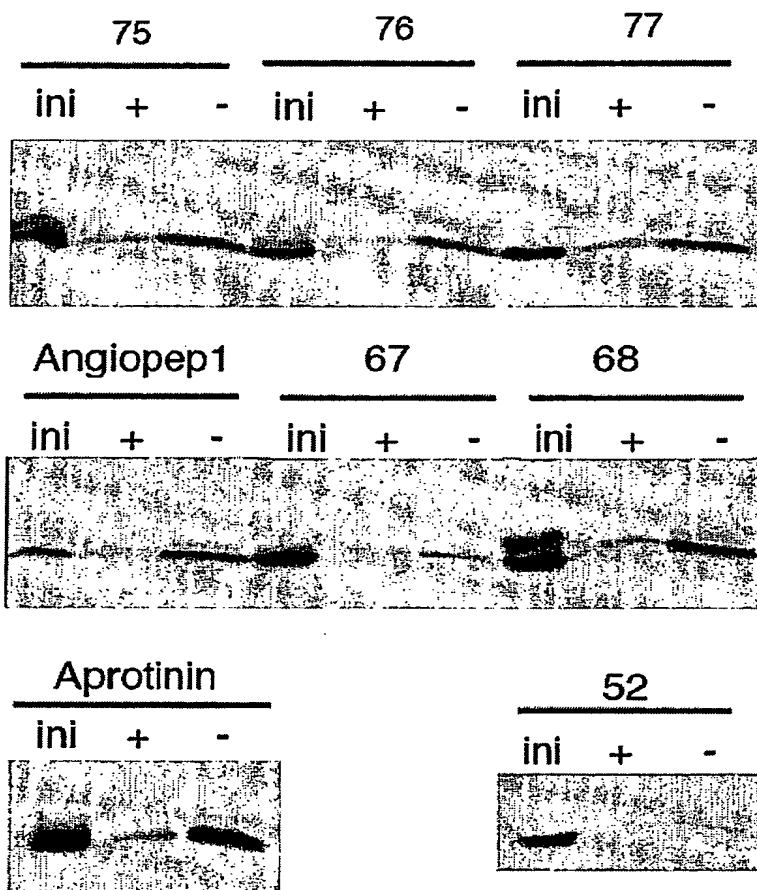


Fig. 1

2 / 9

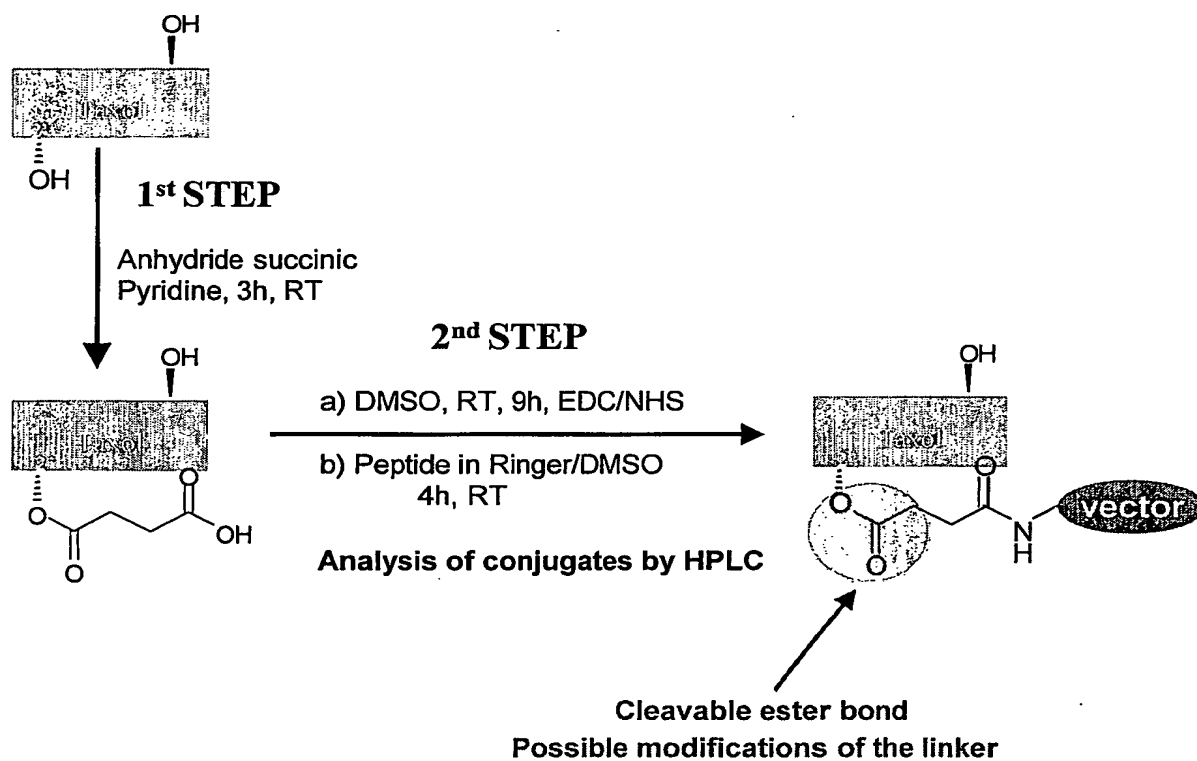


Fig. 2

3 / 9

Survival study 1: CNS-1 glioblastoma model in Lewis rats
Effect of taxol and taxol-aprotinin treatment (IV injection)
(april 04)

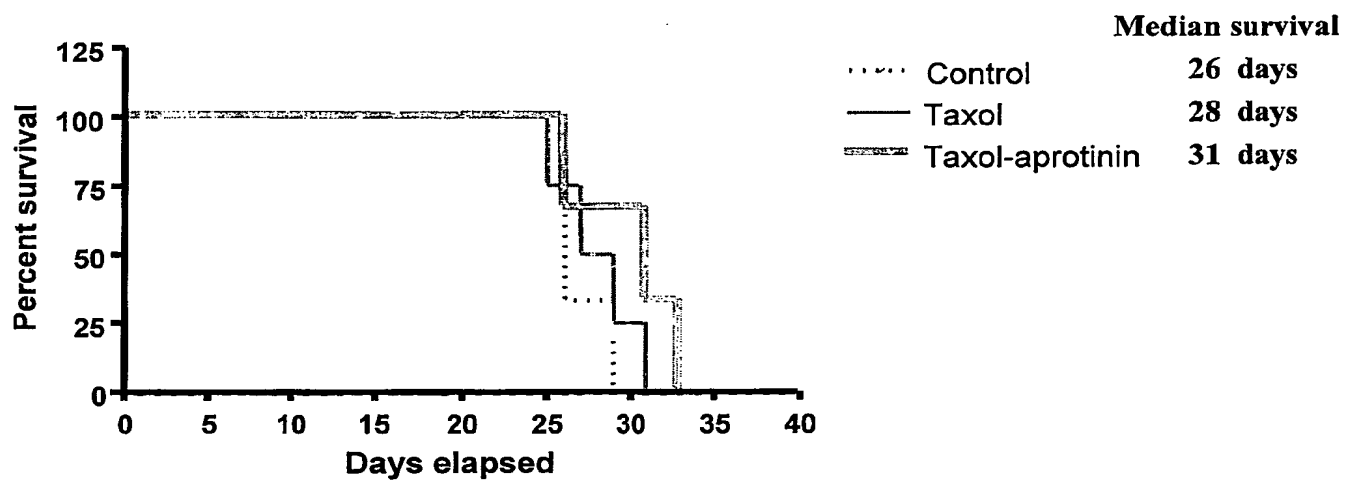


Fig. 3

4 / 9

**Survival study 2: Human U87 glioblastoma model in nude mice . Effect of taxol-Angiopep1 treatment (IV injection)
(May 04)**

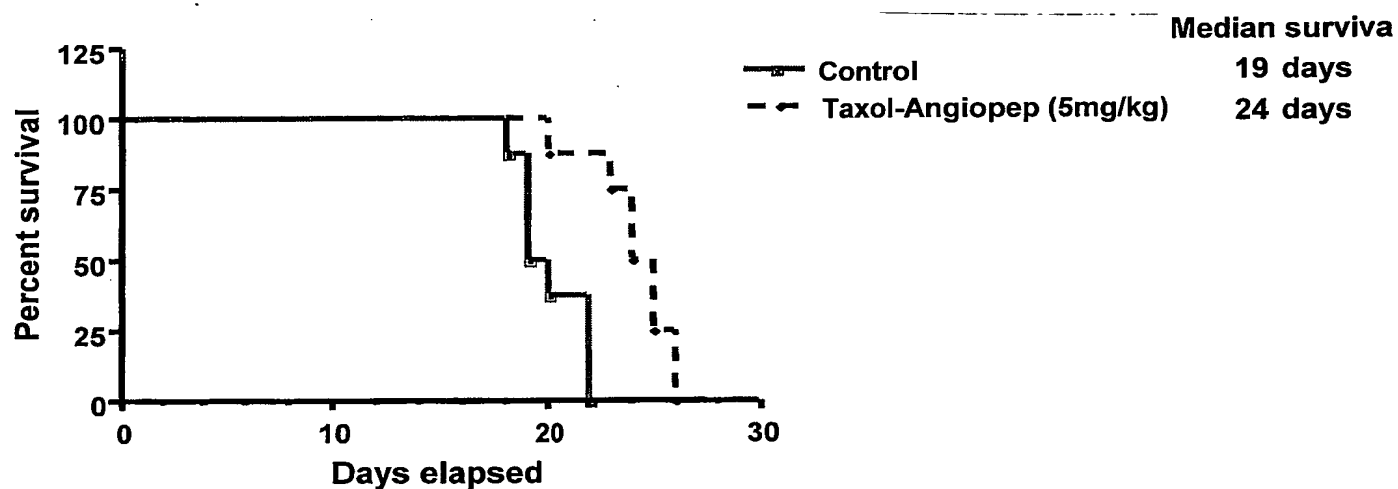


Fig. 4

5 / 9

Cross-linker: BS³

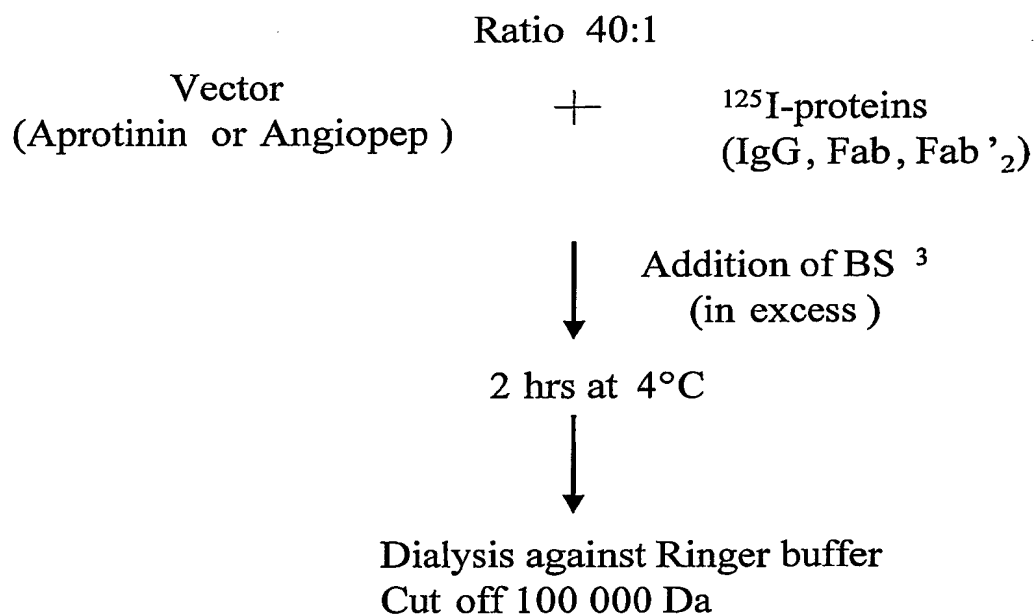


Fig. 5

6 / 9

Cross-linker: sulfo-EMCS

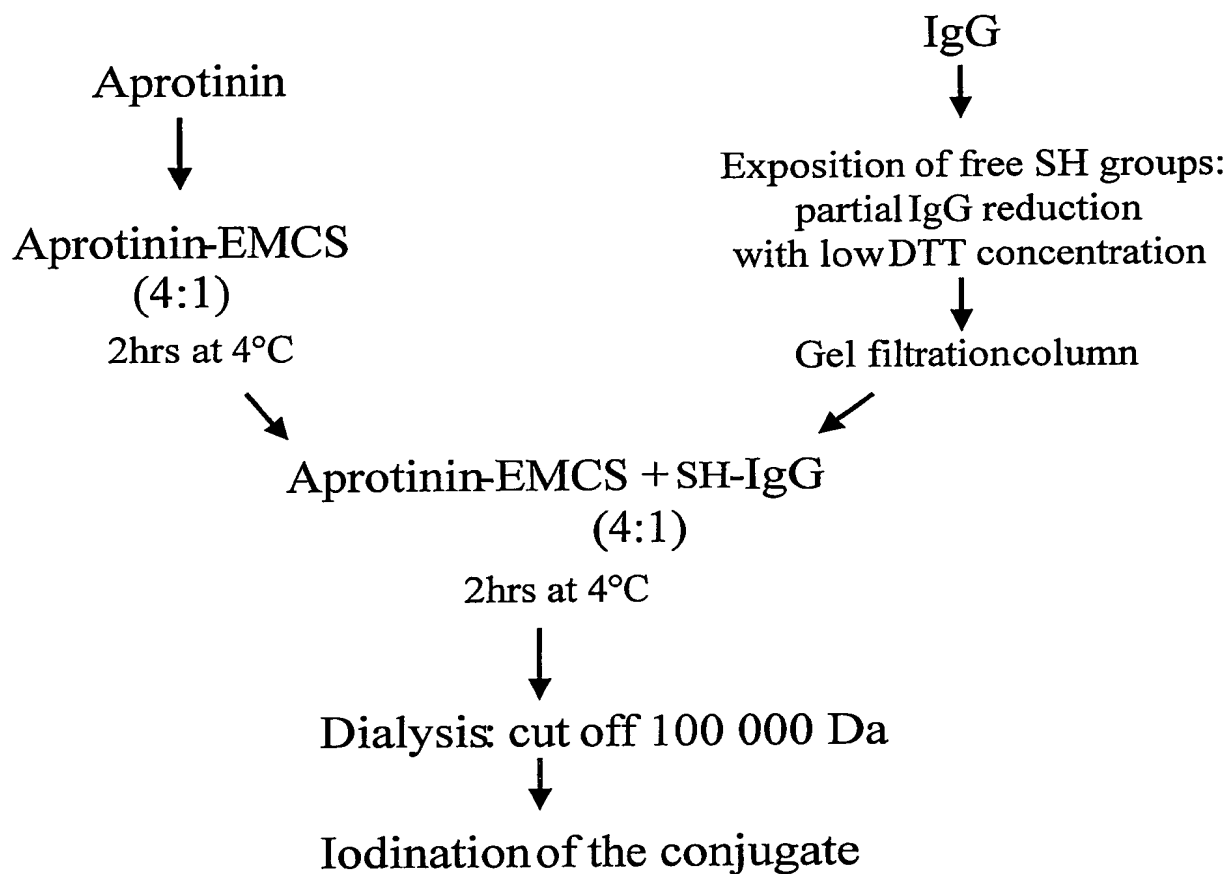
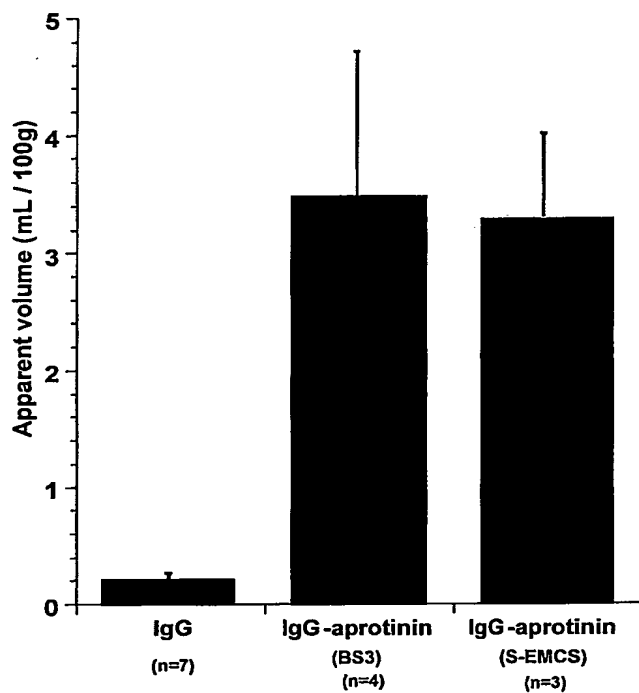


Fig. 6

7 / 9

Higher brain penetration for IgG-aprotinin conjugates

Ratios : IgG / IgG-aprotinin (BS3) = 17.4
IgG / IgG-aprotinin (S-EMCS) = 16

Fig. 7

8 / 9

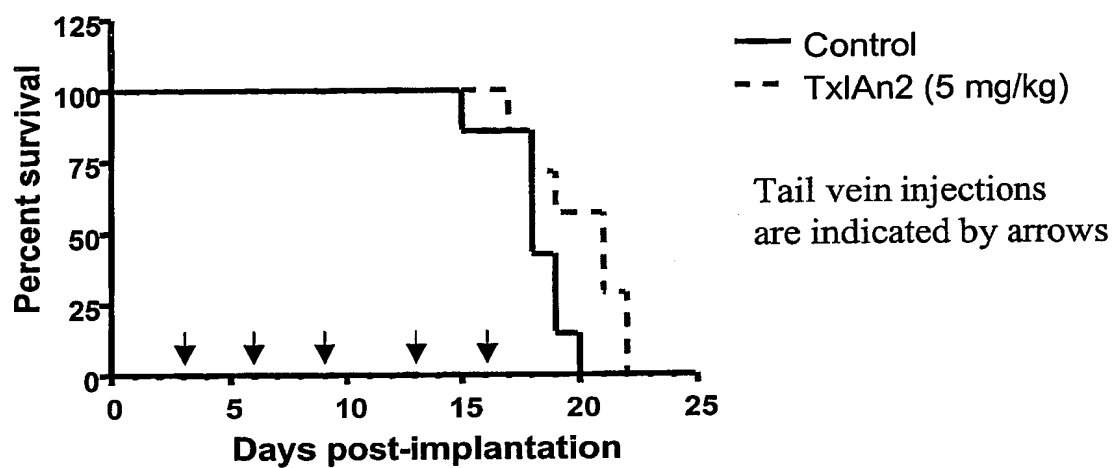
Survival study 4 : Effect of Taxol-Angiopep-2 conjugate (5 mg/kg)

Fig. 8

9 / 9

Peptide	Amino acid sequence	Charge
Angiopep	TFFYGGCRGKRNNFKTEEY	+2
# 67	TFFYGGCRGKRNNFKTEEY-amide	+2
# 76	TFFYGGCRGKRNNFKTKEY-amide	+3
# 91	RFKYGGCLGNKNNYLRLKY-amide	+5
# 5	TFFYGGCRAKRNNFKRAKY-amide	+6

Charge + : lysine (K), arginine (R)

Charge - : glutamic acid (E), aspartic acid (D)

Fig. 9

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2005/001158

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7): C12N 15/15, C07K 14/81, A61K 47/48, A61K 47/42, A61K 51/08, A61K 49/14, A61P 25/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC(7): C12N 15/15, C07K 14/81, A61K 47/48, A61K 47/42, A61K 51/08, A61K 49/14, A61P 25/00

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic database(s) consulted during the international search (name of database(s) and, where practicable, search terms used)

Delphion, Canadian Patent Database, Pubmed, GenomeQuest Keywords: Aprotinin, Angio-pep-1, amyloid B, Kunitz domain, membrane, blood-brain barrier, transport, drug, neurological disease, conjugate

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO2004060403 A2 (TRANSFERT PLUS)	1-5, 9-16, 21, 23-28, 30-37, 39-41, 43, 45, 47-49 and 51-52
Y	22 July 2004 pages 21-35, figures 16-17 and claims cited in application	6-8, 17-20, 22, 29, 38, 42, 44, 46, and 50
X	WO9635788 A2 (SCIOS INC.)	1, 3, 4, 11-16 and 53-54
Y	14 November 1996 whole document	6-8, 17-20, 22, 29, 38, 42, 44, 46, and 50
X	WO9733996 A2 (BAYER CORPORATION)	1, 3, 4, 11-16 and 53-54
Y	18 September 1997 whole document	6-8, 17-20, 22, 29, 38, 42, 44, 46, and 50
X	EP0393431 A1 (BAYER AG) 05 April 1990 whole document	1, 3, 4, 11-16 and 53-54

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents :	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier application or patent but published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

14 November 2005 (14-11-2005)

Date of mailing of the international search report

15 November 2005 (15-11-2005)

Name and mailing address of the ISA/CA
Canadian Intellectual Property Office
Place du Portage I, C114 - 1st Floor, Box PCT
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Gatineau, Quebec K1A 0C9
Facsimile No.: 001(819)953-2476

Authorized officer

Nicole Harris (819) 997-4541

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2005/001158

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of the first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons :

1. ☒ Claim Nos. : 41-44
because they relate to subject matter not required to be searched by this Authority, namely :

Although claims 41-44 encompass methods of treatment and methods of diagnosis of a human or animal which this Authority is not obliged to search under Rule 39.1(iv) of the PCT, the search has been carried out based on the alleged use of the compounds referred to therein.
2. ☐ Claim Nos. :
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically :
3. ☐ Claim Nos. :
because they are dependant claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows :

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claim Nos. :
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim Nos. :

Remark on Protest ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/CA2005/001158

Patent Document Cited in Search Report	Publication Date	Patent Family Member(s)	Publication Date
WO2004060403 A2	22-07-2004	AU2004203682 A1	22-07-2004
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(54) Title: USE OF APROTININ POLYPEPTIDES AS CARRIERS IN PHARMACEUTICAL CONJUGATES

(57) Abstract: Conjugates comprising carriers selected from the group consisting of aprotinin, a biologically active aprotinin fragment, Angiopep-1, Angiopep-2 and biologically active analogs, derivatives or fragments thereof and a label or drug are disclosed. Said conjugates increase the potency of the drug and modify the pharmacokinetics of the drug or label. The use of these conjugates in the treatment and diagnosis of cancer is described.

WO 2007/009229 A1

FIELD OF THE INVENTION

The present invention relates to carriers conjugates and pharmaceutical compositions and their use to increase the potency of drugs and to modify the pharmacokinetics of compounds. More particularly, the present invention relates to conjugates comprising the carrier described herein and their use in the treatment and diagnostic of cancer.

BACKGROUND OF THE INVENTION

Clinical progress in the treatment of primary tumors has been slow and one of the problems associated with these tumors is their weak response to anticancer drugs. The effectiveness of chemotherapy and immunotherapy have been impaired by inherent or acquired multiple drug resistance (MDR) phenotype by cancer cells. One mechanism involved in MDR phenotype is caused by the expression of P-glycoprotein (P-gp), a membrane transporter that pumps out various anticancer drugs from MDR1 expressing cells. P-gp is also expressed in a large number of normal secretory tissues such as kidney, liver and intestine. This efflux pump is strongly expressed in the brain capillaries where its expression was mainly localized in the luminal membrane of endothelial cells lining these. In human, P-gp is encoded by two MDR genes; *MDR1* and *MDR3*. P-gp encoded by the human *MDR1* gene confers the resistance phenotype whereas P-gp encoded by the human *MDR3* gene does not. Thus, P-gp may be seen as a guardian that limits the entry of drugs by expulsing them out of the brain or out of cancer cells preventing them from reaching cytotoxic concentrations.

Cancer cells forming brain metastases originate mostly from lung or breast cancers, colorectal carcinoma, melanoma and urinary organ tumors. These metastases, which often occur after surgery, primary chemotherapy treatment or radiotherapy, are chemo-resistant. Chemotherapy against brain metastases could be effective only if it was effective for their corresponding originate tumors. For example, it was shown that brain metastases originating from small cell lung carcinomas and germ cells respond with similar rates than metastases at other sites.

Drug resistance may be an intrinsic property of tumor cells or may be acquired after treatment. The presence of the P-gp efflux pump encoded by *MDR1* (also herein referred as P-glycoprotein, MDR1 P-gp or MDR1) has been reported in most of the primary brain tumors where most gliomas and more particularly endothelial cells of newly formed capillaries were stained positive for MDR1 P-gp. Thus, various studies support the idea that the multiple drug resistance phenotype may be caused not only by the expression of P-gp in cancer cells but also from its expression in the newly formed endothelial cells in the tumors. MDR1 levels were also found significantly lower in brain metastasis from melanomas and lung adenocarcinomas. In addition, it was shown that treatments prior to surgery have no major impact on MDR1 levels in brain metastasis from melanomas since they were identical in patients that received radiotherapy, chemotherapy or both treatments. In lung metastasis, MDR1 was only detected in patients that received chemotherapy indicating that these previous treatments may have induced its expression resulting in an acquired MDR phenotype. The lack of MDR1 expression in primary lung tumors and in their corresponding brain metastasis indicates also that these metastases did not acquire the same levels of P-gp expression during their development than the ones found in normal brain tissue. These results also indicate that the MDR1 levels of endothelial cells from capillaries in brain metastasis differed from the one of primary brain tumors. The lack of MDR1 expression in some brain metastasis may explain in part why some of them are more sensitive to chemotherapeutic drugs than primary brain tumors.

Methods for transporting a compound across the blood-brain barrier have been described in international application no. PCT/CA2004/000011 published on July 22, 2004 under publication No. WO2004060403, the entire content of which is incorporated herein by reference. Briefly, in this document, aprotinin, aprotinin fragments and analogs were presented as a drug delivery system for the central nervous system (CNS) and for treating CNS related diseases.

There remains a need for increasing the potency of anticancer drugs.

The present invention seeks to meet these and other needs.

SUMMARY OF THE INVENTION

5 The present invention relates in one aspect thereof, to a carrier comprising an amino acid sequence selected from the group consisting of the amino acid sequence of aprotinin, a biologically active aprotinin fragment, Angiotensin-1, Angiotensin-2 and biologically active analogs, derivatives or fragments thereof. The aprotinin sequence as well as some exemplary embodiments of biologically active analogs may be found for example in international application no. PCT/CA2004/000011.

10 The present invention also relates to a carrier consisting of an amino acid sequence selected from the group consisting of the amino acid sequence of aprotinin, a biologically active aprotinin fragment, Angiotensin-1, Angiotensin-2 and biologically active analogs, derivatives or fragments thereof.

15 Exemplary embodiment of carriers encompassed by the present invention includes those which may be selected, for example, from the group consisting of

- aprotinin (SEQ ID NO.:98),
- an aprotinin analogue
- 20 - an aprotinin fragment which may comprise (or may consist essentially of) the amino acid sequence defined in SEQ ID NO.:1,
- a biologically active analogue of SEQ ID NO.:1,
- a biologically active fragment of SEQ ID NO.:1, and;
- a biologically active fragment of a SEQ ID NO.:1 analogue.

25 More particularly, the carrier may be selected, for example, from the group of;

- an aprotinin fragment which may comprise the amino acid sequence defined in SEQ ID NO.:1,
- a biologically active analogue of SEQ ID NO.:1,
- 30 - a biologically active fragment of SEQ ID NO.:1 and;
- a biologically active fragment of a SEQ ID NO.:1 analogue.

In accordance with the present invention the aprotinin fragment may consist of the sequence defined in SEQ ID NO.:1. Further in accordance with the present invention, the aprotinin fragment may comprise SEQ ID NO.1 and may have a length of from about 19 amino acids to about 54 amino acids, e.g., from 10 to 50 amino acids in length, from 10 to 30 amino acids in length etc.

In accordance with the present invention, the biologically active analogue of SEQ ID NO.:1, may have a length of from about 19 amino acids to about 54 amino acids (e.g., including for example 21 to 23, 25 to 34, 36 to 50 and 52 to 54), or of from about 19 amino acids to about 50 amino acids, or from about 19 amino acids to about 34 amino acids (e.g., 19, 20, 21, 22, 23, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34), of from about 19 amino acids to about 23 amino acids or of about 19, 20, 21, 22, 23, 24, 35, 51, amino acids.

The polypeptides of the present invention may be amidated, i.e., may have an amidated amino acid sequence.

A biologically active fragment of a polypeptide (e.g., of 19 amino acids) described herein may include for example a polypeptide of from about 7, 8, 9 or 10 to 18 amino acids.

Therefore, in accordance with the present invention, a biologically active fragment of SEQ ID NO.:1 or of a SEQ ID NO.:1 analogue may have a length of from about 7 to about 18 amino acids or from about 10 to about 18 amino acids.

U.S. patent no. 5,807,980 describes a polypeptide which is identified herein as SEQ ID NO.:102.

U.S. Patent no. 5,780,265 describes a polypeptide which is identified herein as SEQ ID NO.:103.

The aprotinin amino acid sequence (SEQ ID NO.:98), the Angiopep-1 amino acid sequence (SEQ ID NO.:67), as well as some sequences of biologically active analogs may be found for example in international application no. PCT/CA2004/000011 published on July 22, 2004 in under international publication no. WO2004/060403. Additionally, international publication No. WO04/060403 describes a polypeptide which is identified herein as SEQ ID NO.: 104.

U.S. Patent no.5,118,668 describes polypeptides which has the sequence illustrated in SEQ ID NO.: 105.

- 5 Even more particularly, the carrier may be selected, for example, from the group of;
- a SEQ ID NO.:1 analogue which may comprise at least 35 % identity with the amino acid sequence of SEQ ID NO.:1,
 - a SEQ ID NO.:1 analogue which may comprise at least 40 % identity with the amino acid sequence of SEQ ID NO.:1,
 - 10 - a SEQ ID NO.:1 analogue which may comprise at least 50 % identity with the amino acid sequence of SEQ ID NO.:1,
 - a SEQ ID NO.:1 analogue which may comprise at least 60 % identity with the amino acid sequence of SEQ ID NO.:1,
 - a SEQ ID NO.:1 analogue which may comprise at least 70 % identity with the amino acid sequence of SEQ ID NO.:1,
 - 15 - a SEQ ID NO.:1 analogue which may comprise at least 80 % identity with the amino acid sequence of SEQ ID NO.:1,
 - a SEQ ID NO.:1 analogue which may comprise at least 90 % identity with the amino acid sequence of SEQ ID NO.:1 and;
 - 20 - a SEQ ID NO.:1 analogue which may comprise at least 95 % (i.e., 96%, 97%, 98%, 99% and 100%) identity with the amino acid sequence of SEQ ID NO.:1.

For example, the biologically active analogue of SEQ ID NO.:1 may comprise an amino acid sequence selected from the group consisting of an amino acid sequence defined in any one of
25 SEQ ID NO.:2 to SEQ ID NO.: 62, SEQ ID NO.: 68 to SEQ ID NO.: 93, and SEQ ID NO.:97 as well as 99, 100 and 101.

Further in accordance with the present invention, the biologically active analogue of SEQ ID NO.:1 may comprise the amino acid sequence defined in SEQ ID NO.:67. This sequence
30 may more particularly be amidated.

For example and without limitation, conjugates comprising peptides SEQ ID NO.: 102, 103, 104 and 105 are also encompassed by the present invention.

Further in accordance with the present invention, the biologically active fragment of SEQ ID NO.:1 or the biologically active fragment of a SEQ ID NO.:1 analogue may comprise at least 9 or at least 10 (consecutive or contiguous) amino acids of SEQ ID NO.1 or of the SEQ ID NO.:1 analogue.

The polypeptides of the present invention may have an amino acid sequence which may comprise of from between 1 to 12 amino acid substitutions (i.e., SEQ ID NO.:91). For example, the amino acid substitution may be from between 1 to 10 amino acid substitutions, or from 1 to 5 amino acid substitutions. In accordance with the present invention, the amino acid substitution may be a non-conservative amino acid substitution or a conservative amino acid substitution.

For example, when a polypeptide of the present invention comprises amino acids which are identical to those of SEQ ID NO.:1 and other amino acids which are not identical (non-identical), those which are non-identical may be a conservative amino acid substitution. The comparison of identical and non-identical amino acids may be performed by looking at a corresponding location.

Examples of SEQ ID NO.:1 analogue which may have at least 35% identity includes for example, a polypeptide comprising (consisting of) the amino acid sequence defined in SEQ ID NO.:91 (about 36.8% identity, i.e., 7 amino acid out of 19 amino acids of SEQ ID NO.:91 are identical to SEQ ID NO.:1), a polypeptide comprising (consisting of) the amino acid sequence defined in SEQ ID NO.:98 (about 68.4% identity, i.e., 13 amino acid out of 19 amino acids are identical to SEQ ID NO.:1), a polypeptide comprising (consisting of) the amino acid sequence defined in SEQ ID NO.:67 (about 73.7% identity, i.e., 14 amino acid out of 19 amino acids are identical to SEQ ID NO.:1), a polypeptide comprising (consisting of) the amino acid sequence defined in SEQ ID NO.: 76 (about 73.7% identity, i.e., 14 amino acid out of 19 amino acids are identical to SEQ ID NO.:1) and a polypeptide comprising (consisting of) the amino acid sequence defined in SEQ ID NO.:5 (about 79 % identity, i.e., 15 amino acid out of 19 amino acids are identical to SEQ ID NO.:1).

Examples of SEQ ID NO.:1 analogue which may have at least 60% identity includes for example, a polypeptide comprising (consisting of) the amino acid sequence defined in SEQ ID NO.:98 (about 68.4% identity, i.e., 13 amino acid out of 19 amino acids are identical to SEQ ID NO.:1), a polypeptide comprising (consisting of) the amino acid sequence defined in
5 SEQ ID NO.:67 (about 73.7% identity, i.e., 14 amino acid out of 19 amino acids are identical to SEQ ID NO.:1), a polypeptide comprising (consisting of) the amino acid sequence defined in SEQ ID NO.: 76 (about 73.7% identity, i.e., 14 amino acid out of 19 amino acids are identical to SEQ ID NO.:1) and a polypeptide comprising (consisting of) the amino acid
10 sequence defined in SEQ ID NO.:5 (about 79 % identity, i.e., 15 amino acid out of 19 amino acids are identical to SEQ ID NO.:1).

Examples of SEQ ID NO.:1 analogue which may have at least 70% identity includes for example, a polypeptide comprising (consisting of) the amino acid sequence defined in SEQ ID NO.:67 (about 73.7% identity, i.e., 14 amino acid out of 19 amino acids are identical to
15 SEQ ID NO.:1), SEQ ID NO.: 76 (about 73.7% identity, i.e., 14 amino acid out of 19 amino acids are identical to SEQ ID NO.:1), SEQ ID NO.:5 (about 79 % identity, i.e., 15 amino acid out of 19 amino acids are identical to SEQ ID NO.:1).

In accordance, with the present invention, the carrier may more particularly be selected from
20 the group consisting of peptide Nos. 5, 67, 76, 91 and peptide 97 (i.e., SEQ ID NO.:5, 67, 76, 91 and 97 (Angiopep-2)).

The present invention particularly relates to the use of a carrier or the pharmaceutical composition described herein for modifying and/or improving the (*in vivo*) pharmacokinetics
25 of a compound.

In accordance with the present invention, the compound may be selected, for example, from the group consisting of a label, a protein, a peptide and a small molecule drug and combination thereof.
30

Also in accordance with the present invention, the small molecule drug may be, for example, an anticancer drug.

In accordance with the present invention the anticancer drug may be conjugated with the carrier thereby forming a conjugate. In an exemplary embodiment of the invention, the conjugate may comprise, for example, at least one anticancer drug molecule for each carrier molecule. In another exemplary embodiment of the invention, the conjugate may comprise,
5 for example, at least two anticancer drug molecules for each carrier molecule. In yet another exemplary embodiment of the invention, the conjugate may comprise, for example, at least three anticancer drug molecules for each carrier molecule.

10 In accordance with the present invention the carrier may promote accumulation of the drug in a tissue such as, for example, a kidney (kidney tissue), a liver (liver tissue), an eye (eye tissue) and the lungs (lung tissue) of an individual.

Also in accordance with the present invention, the carrier may modify or improve the bioavailability of the compound.

15

Further in accordance with the present invention, the carrier may also change the (usual) tissue distribution of the compound.

20 In accordance with the present invention the carrier may also promote accumulation of the drug in the brain (brain tissue) of an individual.

In accordance with the present invention, the brain may be a tumoral brain.

Further in accordance with the present invention, the brain may comprise a lung cancer cell.

25

Also in accordance with the present invention, the carrier may promote accumulation of the drug in a cancer cell (e.g., intracellular accumulation of the drug in the cancer cell).

30

As used herein the term "tumoral brain" refers to a brain which comprises a tumor, either a primary tumor or a metastasis of a different tissue origin, such as, without limitation, a metastasis originating from a lung tumor, a breast tumor, from a melanoma, from a colorectal tumor, from a tumor of an urinary organ or else. Examples of tumoral brain cells

thus include, for example, glioblastomas, and metastatic cell originating, for example, from the lung, breast, colon, urinary tract or from melanoma.

- 5 In accordance with the present invention, the carrier may thus be used, for example, for reducing the dose of a drug, necessary to achieve the same therapeutic effect (e.g., to achieve a reduction in tumor cell growth, etc.).

10 The present invention further relates to the use of a carrier selected from the group consisting of aprotinin, a biologically active aprotinin fragment, Angiopep-1, Angiopep-2, biologically active analogs, derivatives or fragments and combination thereof for transporting a compound to a desired target site, a desired target tissue or a desired target cell.

15 Examples of a small molecule drug which may be conjugated with the carrier of the present invention and which are encompassed herewith, includes for example and without limitation, Taxol, a Taxol derivative, vinblastine, vincristine, etoposide, doxorubicin, cyclophosphamide, Taxotere, melphalan, chlorambucil, pharmaceutically acceptable salts, etc. and combination thereof as well as any drug which may be a P-gp substrate.

20 Other small molecule drug encompassed by the present invention may include, for example, a drug having a group allowing it's conjugation to the carrier of the present invention.

In accordance with the present invention, exemplary embodiments of Taxol derivatives (or analogues) include for example, derivatives disclosed and referred to in U.S. Patent No.
25 6,911,549 issued on June 28, 2005, the entire contents of which is incorporated herein by reference.

30 Examples of labels which may be conjugated with the carrier of the present invention and which are encompassed herewith include, for example and without limitation, an isotope, a fluorescent label (e.g., rhodamine), a reporter molecule (e.g., biotin), etc.

Examples of protein which may be conjugated with the carrier of the present invention and which are encompassed herewith includes, without limitation, an antibody, an antibody

fragment, a peptidic- or protein-based drug (e.g., a positive pharmacological modulator (agonist) or an pharmacological inhibitor (antagonist)) etc.

5 The present invention also provides the use of a carrier selected from the group consisting of aprotinin, a biologically active aprotinin fragment, Angiopep-1, Angiopep-2, biologically active analogs, derivatives or fragments and combination thereof for increasing the potency of a drug. More particularly, the carrier may be used to increase, for example, the potency of a drug which may be a P-gp substrate or drugs which are expelled (i.e., expelled, ejected from a cell, etc.) by P-gp or P-gp related protein (e.g., a P-gp human or mammalian allelic variant, e.g., a mdrla and/or mdrlb isoforms from a rodent, etc.).

10

In accordance with the present invention, the carrier may increase for example, the potency of an anticancer drug, for example, an anticancer drug which may be P-gp substrates.

15 In yet an additional aspect, the present invention more particularly relates to the use of a carrier, conjugate or the pharmaceutical composition described herein for increasing (optimizing) an anti-tumor growth effect of an anticancer drug.

The present invention further provides the use of a carrier selected from the group consisting of aprotinin, a biologically active aprotinin fragment, Angiopep-1, Angiopep-2, biologically active analogs, derivatives or fragments and combination thereof for transporting a drug or a label at or to a desired target site or for transporting a drug or a label inside a target cell and/or for promoting the accumulation of a drug or a label inside a cell, such as for example, a cell which expresses P-gp at its surface or a cell which is able to express P-gp (at its surface).

20

25

The carrier may be used, for example, for promoting the accumulation, inside a cell, of a drug which may comprise the characteristic of being expelled (i.e., expelled, transported outside a cell, ejected) by P-gp or a P-gp related protein.

30

In accordance with the present invention, the desired site may be, for example and without limitation, the brain or other sites outside the brain (e.g., an extracranial site) such as for example, the kidney, the liver, the pancreas, the colon, the eyes, the lungs and combination

thereof. Therefore, the desired target site may be one or more site selected from the group consisting of the brain, the kidney, the liver, the pancreas, the colon, the eyes, the lungs and combination thereof.

- 5 In accordance with a particular embodiment of the present invention, a desired target site may be, for example, a brain cell or tissue.

In accordance with another particular embodiment of the present invention, a desired target site may be, for example, a liver cell or tissue.

10

In accordance with a further particular embodiment of the present invention, a desired target site may be for example, a kidney cell or tissue.

- 15 In accordance with yet a further particular embodiment of the present invention, a desired target site may be for example, a pancreas cell or tissue.

In accordance with another particular embodiment of the present invention, a desired target site may be for example, a colon cell or tissue.

- 20 In accordance with yet another particular embodiment of the present invention, a desired target site may be for example, eye or an eye cell.

In accordance with a further particular embodiment of the present invention, a desired target site may be for example, a lung cell or tissue.

25

Further in accordance with the present invention, the desired site may be a site which comprises a cell expressing a carrier receptor or transporter, for example, a cell expressing a low-density lipoprotein related receptor (LRP). The cell may also be a cell which co-expresses P-gp or a P-gp related protein. The cell may be, for example a normal cell, a
30 tumor cell, or a metastatic cell. The carrier of the present invention may thus be used to target a brain cell, a liver cell, a kidney cell, a pancreas cell, a colon cell, an eye cell, a lung cell and combination thereof (either normal or tumoral).

The present invention also relates in a more particular aspect thereof to the use of a carrier described herein (e.g., which may be selected from the group consisting of aprotinin, a biologically active aprotinin fragment, Angiopep-1, Angiopep-2 and biologically active analogs, derivatives or fragments thereof) or the pharmaceutical composition or the conjugate described herein for promoting intracellular accumulation of a compound (i.e., promoting accumulation of the compound inside a cell).

In accordance with an embodiment of the present invention, the compound may be selected, for example, from the group consisting of a label, a protein, a peptide and a small molecule drug.

In accordance with a further embodiment of the present invention, the cell may be a cell which is able to express P-gp or which expresses P-gp. More particularly the cell may express P-gp (MDR1) at the cell surface.

The cell may be, for example, a tumor cell. The tumor cell may originate for example and without limitation, from a brain tumor, a lung tumor, a breast tumor, a kidney tumor, an eye tumor, a liver tumor, a colorectal tumor, a tumor of an urinary organ, etc.

In accordance with the present invention the cell may be located outside of a brain of an individual (mammal, animal, etc.). For example, the cell may be a tumor cell which may be located outside of a brain of an individual (mammal, animal, etc.).

In accordance with a further embodiment of the present invention, the cell may be located inside a brain of an individual. The cell may be, for example, a tumor cell which may be located inside of a brain of an individual (mammal, animal, etc.).

In an exemplary embodiment of the present invention, the tumor cell may be a brain tumor cell. For example, the brain tumor cell may originate from a glioblastoma or may be a glioblastoma.

In another exemplary embodiment of the present invention, the tumor cell may be a lung tumor cell.

The present invention also relates in an additional aspect thereof to the use of the carrier, the conjugate or the pharmaceutical composition described herein for reducing the elimination of a drug from the inside of a cell, such as for example a cell which may be able to express P-gp (MDR1) or which expresses P-gp. In accordance with the present invention, the drug may be a P-gp substrate.

Also in accordance with the present invention, the cell may be a multiple drug resistant cancer cell.

In yet an additional aspect thereof, the present invention relates to the use of a carrier, the conjugate or the pharmaceutical composition described herein for reducing the growth of a cell. For that purpose, the carrier may be conjugated with a drug which may be able to reduce the growth of a cell.

In accordance with a non-limitative exemplary embodiment of the invention, the carrier, the conjugate thus formed or the pharmaceutical composition may be used to reduce the growth of a tumor cell or an endothelial cell.

In a particular embodiment of the invention, the tumor cell may be able to express or expresses P-gp (MDR1).

In an exemplary embodiment of the invention, the tumor cell may be a brain tumor cell. More specifically, the brain tumor cell may originate from a glioblastoma or may be a glioblastoma.

In another exemplary embodiment of the invention, the tumor cell may be a lung tumor cell.

In yet another exemplary embodiment of the invention, the tumor cell may be a breast tumor cell.

In a further exemplary embodiment of the invention, the tumor cell may be a kidney tumor cell.

In yet a further exemplary embodiment of the invention, the tumor cell may be an eye tumor cell.

- 5 In an additional embodiment of the invention, the tumor cell may be from a colorectal cancer.

In another additional embodiment of the invention, the tumor cell may be from a urinary organ tumor.

10

In a particular embodiment of the invention, the anticancer drug may, more specifically be Taxol, Taxotere or a Taxol or Taxotere derivative.

- 15 In accordance with another embodiment of the present invention, the anticancer drug may be, for example, vinblastine.

In accordance with yet another embodiment of the present invention, the anticancer drug may be, for example, vincristine.

- 20 In accordance with a further embodiment of the present invention, the anticancer drug may be, for example, etoposide.

In accordance with a further embodiment of the present invention, the anticancer drug may be, for example, doxorubicin.

25

In accordance with an additional embodiment of the present invention, the anticancer drug may be, for example, cyclophosphamide.

- 30 In accordance with yet an additional embodiment of the present invention, the anticancer drug may be, for example, melphalan.

In accordance with yet another embodiment of the present invention, the anticancer drug may be, for example chlorambucil.

In another aspect, the present invention relates to the use of a carrier described herein in the making of a pharmaceutical composition or medicament for modifying the pharmacokinetics of the small molecule drug.

5

More particularly the carrier may be used for reducing the growth of a cell.

Also more particularly, the carrier may be used for promoting accumulation of the small molecule drug inside a cell.

10

In addition the carrier may be used for reducing the elimination of the small molecule drug from the inside of a cell.

Also, the carrier may be used for increasing an anti-tumor growth effect of the small molecule drug.

15

Furthermore, the carrier may be used to improve the bioavailability of the small molecule drug.

20

In addition and in accordance with the present invention, the carrier may be used to change the (usual) tissue distribution of the small molecule drug.

In addition, the present invention relates to the use of a carrier selected from the group consisting of aprotinin, a biologically active aprotinin fragment, Angiopep-1, Angiopep-2, biologically active analog and combination thereof for treating cancer, metastatic cancer and/or metastasis. In accordance with the present invention, an exemplary metastasis may comprise, without limitation, a metastasis which may originate from a breast tumor, a lung tumor, a melanoma, etc.

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30

The present invention also relates to the use of a carrier selected from the group consisting of aprotinin, a biologically active aprotinin fragment, Angiopep-1, Angiopep-2, biologically active analogs, derivatives or fragments and combination thereof for the detection of a desired target cell.

The present invention further provides the use of a carrier selected from the group consisting of aprotinin, a biologically active aprotinin fragment, Angiopep-1, Angiopep-2, biologically active analogs, derivatives or fragments and combination thereof for the diagnostic of a cancer, a metastatic cancer and/or metastasis.

The present invention additionally relates in a further aspect, to a composition (e.g., pharmaceutical composition) comprising a carrier (and/or pharmaceutically acceptable salt thereof) of the present invention and a pharmaceutically acceptable carrier.

The present invention in an additional aspect relates to a conjugate and/or pharmaceutically acceptable salt thereof. The conjugate may comprise, for example, a carrier as described herein and a drug, a label or a protein. The carrier may be covalently attached to the drug, label, protein or peptide.

More particularly, the conjugate may comprise, for example a carrier selected from the group consisting of aprotinin, a biologically active aprotinin fragment, Angiopep-1, Angiopep-2, biologically active analogs, derivatives or fragments and combination thereof and a compound selected from the group consisting of a drug, a label, a protein and combination thereof. In accordance with the present invention, the conjugate may comprise one or more drug molecules.

Also in accordance with the present invention, the compound may or may not be released from the carrier. The compound may therefore be releasable from the conjugate (or from the carrier).

In accordance with the present invention, the conjugate may comprise the formula R-L-M wherein R is a class of molecules related to aprotinin (e.g., aprotinin, aprotinin fragment, Angiopep-1, Angiopep-2, analogs, derivatives or fragments), L may be a linker or a bond and M may be an agent or a drug selected from the group consisting of a drug (e.g., a small molecule drug), a label, a protein (e.g., antibody, an antibody fragment) and a polypeptide. It is to be understood herein that the formula R-L-M is not intended to be restricted to a

specific order or specific ratio. As being exemplified herein, M may be found in several ratios over R.

The present invention relates in a further aspect thereof to the use of a conjugate which may
5 comprise a) a carrier which may be selected from the group consisting of aprotinin, a biologically active aprotinin fragment, Angiopep-1, Angiopep-2 and biologically active analogs, derivatives or fragments thereof and b) at least one small molecule drug or label for modifying the pharmacokinetics (*in vivo*) of the drug or label which is attached thereto.

10 In accordance with one embodiment of the present invention, the conjugate may be, more particularly, used for reducing the growth of a cell.

Further in accordance with the present invention, the conjugate may be used for promoting accumulation of the small molecule drug or label inside a cell.

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Also in accordance with the present invention, the conjugate may also be used for reducing the elimination of the small molecule drug or label from the inside of a cell.

Further in accordance with the present invention, the conjugate may be used for increasing
20 an anti-tumor growth effect of the small molecule drug.

Also in accordance with the present invention, the conjugate may improve the bioavailability of the compound.

25 Further in accordance with the present invention, the conjugate may also change the tissue distribution of the compound.

In one embodiment of the invention, the small molecule drug may be able to reduce the growth of a cell.

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The present invention also provides in an additional aspect thereof for the use of a conjugate which may comprise a) a carrier which may be selected, for example, from the group consisting of aprotinin, a biologically active aprotinin fragment, Angiopep-1, Angiopep-2

and biologically active analogs, derivatives or fragments thereof and **b)** at least one small molecule drug in the making (manufacture) of a pharmaceutical composition or medicament for modifying the pharmacokinetics of the small molecule drug.

- 5 The present invention also relates to the use of the carrier in the manufacture of a composition or medicament for the treatment of a condition such as cancer, metastatic cancer and/or metastasis.

10 The present invention also provides in an additional aspect thereof for the use of a conjugate which may comprise **a)** a carrier which may be selected, for example, from the group consisting of aprotinin, a biologically active aprotinin fragment, Angiopep-1, Angiopep-2 and biologically active analogs, derivatives or fragments thereof and **b)** at least one label, for the detection of a cell which may be selected, for example, from the group consisting of an eye cell, a brain cell, a breast cell, a liver cell a kidney cell, a urinary organ cell, a colon cell,
15 a cell from the rectum and a lung cell.

In a particular embodiment of the present invention, the cell which may be detected may be, for example, an eye cell.

- 20 In another particular embodiment of the present invention, the cell which may be detected may be, for example, a brain cell.

In an additional particular embodiment of the present invention, the cell which may be detected may be, for example, a liver cell.

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In yet an additional particular embodiment of the present invention, the cell which may be detected may be, for example, a breast cell.

- 30 In another particular embodiment of the present invention, the cell which may be detected may be, for example, a kidney cell.

In yet another particular embodiment of the present invention, the cell which may be detected may be, for example, lung cell.

The present invention additionally relates to a composition (e.g., pharmaceutical composition) comprising a conjugate of the present invention and a pharmaceutically acceptable carrier.

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The pharmaceutical composition described herein may be used, for example, in the treatment or detection of cancer, a metastatic cancer and/or metastasis.

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The present invention provides in a particular aspect thereof, a pharmaceutical composition which may be able, for example, of reducing the growth of a cell or for the detection of a cell, the pharmaceutical composition may comprise:

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- a) a conjugate which may comprise a carrier selected from the group consisting of aprotinin, a biologically active aprotinin fragment, Angiopep-1, Angiopep-2 and biologically active analogs, derivatives or fragments thereof and a label or a small molecule drug able to reduce the growth of a cell and;
- b) a pharmaceutically acceptable carrier.

The present invention also provides in a further aspect thereof, a pharmaceutical composition which may comprise:

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- a) a conjugate which may comprise a carrier selected from the group consisting of aprotinin, a biologically active aprotinin fragment, Angiopep-1, Angiopep-2 and biologically active analogs, derivatives or fragments thereof and a small molecule drug or a label,
- b) a pharmaceutically acceptable carrier, and;
- c) a solubilizer.

In accordance with the present invention, the solubilizer may be, for example, a poly-oxyethylene ester of fatty acid. Solubilizers encompassed by the present invention, include, for example, Solutol® HS-15.

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An exemplary embodiment of the present invention, a suitable solubilizer may comprise, without limitation, a poly-oxyethylene ester of fatty acid such as for example, Solutol® HS-15.

In accordance with an embodiment of the invention, the pharmaceutical composition may be used more specifically for modifying the pharmacokinetics of a compound, for reducing the growth of a tumor cell or for the detection of a tumor cell, etc.

5

The present invention further relates to the use of at least one conjugate of the present invention for treating cancer, metastatic cancer and/or metastasis. In accordance with the present invention, an exemplary metastasis which may be treated using the conjugate of the present invention is a metastasis which may originate, for example and without limitation

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from a breast tumor, a lung tumor, a melanoma etc.

The present invention also provides in another aspect thereof, a method for treating a cancer (such as, for example, a primary tumor or a metastatic cancer and/or a metastasis) or for detecting a cancer cell, the method may comprise administering an individual with a

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pharmaceutical composition described herein or with a conjugate which may comprise a) a carrier which may be selected from the group consisting of aprotinin, a biologically active aprotinin fragment, Angiopep-1, Angiopep-2 and biologically active analogs, derivatives or fragments thereof and b) an anticancer drug or a label.

20

In accordance with the present invention, the individual may have, for example, an extracranial tumor, a primary brain tumor or a brain tumor of metastatic origin.

The method may also comprise a step of assessing whether the tumor of the individual comprises a multiple drug resistant tumor cell or for example a cell expressing P-gp (MDR1)

25

or determining whether the tumor may have or has a multiple resistance drug phenotype.

The individual may be one which has a tumor. The individual may also be one which has received or which will receive chemotherapy or radiotherapy or both. The individual may also be one who has had surgery. Furthermore, the individual may be one that has a brain tumor or may also be one who has a tumor at another site than brain (is free of a brain

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tumor). An individual in need may also be, for example, an individual which present or is at risk of presenting, a resistance (a multiple drug resistance (MDR) phenotype) to at least one drug.

More particularly and in accordance with the present invention, the individual may have, for example, an extracranial tumor.

- 5 In accordance with the present invention, the extracranial tumor may be, for example, a lung tumor.

- Also in accordance with the present invention, the extracranial tumor may be, for example, an extracranial metastasis from a brain tumor. In a more specific embodiment of the present
10 invention, the extracranial brain tumor may be, for example, a glioblastoma (extracranial metastasis from glioblastoma).

- In another particular embodiment of the present invention, the individual may have a brain tumor of metastatic origin. The brain tumor of metastatic origin may originate, for example,
15 from a lung tumor. The brain tumor of metastatic origin may also originate, for example, from a breast tumor. Additionally, the brain tumor of metastatic origin may also originate, for example, from a melanoma. Furthermore and as described herein, the brain tumor of metastatic origin may also originate, for example, from a colorectal cancer. In addition, the brain tumor of metastatic origin may also originate, for example, from a urinary organ
20 tumor.

In accordance with the present invention, the tumor may comprise a tumor cell which may be able to express P-gp or which expresses P-gp.

- 25 In accordance with the present invention the tumor may comprise a tumor cell which may be able to express LRP or which expresses LRP.

- In accordance with the present invention, the tumor cell may comprise a tumor cell which may be able to co-express P-gp and LRP or which co-expresses P-gp and LRP. P-gp and
30 LRP may be located, for example, at a cell surface.

More particularly, the present invention in an aspect thereof, relates to a method of promoting accumulation of a drug in the brain of an individual having a metastasis. The

method may comprise administering a carrier or conjugate as described herein to an individual having a brain metastasis. In accordance with the present invention, the metastasis may originate from a lung cancer, etc.

- 5 The present invention provides in an additional aspect thereof, a method for promoting intracellular accumulation of a compound selected from the group consisting of a label, a protein, a peptide and a small molecule drug.

- 10 The method may comprise the step of contacting the cell or providing the cell with a conjugate which may comprise a) a carrier which may be selected from the group consisting of aprotinin, a biologically active aprotinin fragment, Angiotensin-1, Angiotensin-2 and biologically active analogs, derivatives or fragments thereof and b) the desired compound.

- 15 In accordance with the present invention, the cell may be, for example, a cell expressing P-gp.

Further in accordance with the present invention, the cell may be, for example, a brain cell, a lung cell, a breast cell, a kidney cell, an eye cell or a liver cell.

- 20 Also in accordance with the present invention, the cell may be, for example, a tumor cell which may be located outside of a brain of an individual (mammal, animal, etc.).

- 25 The present invention also provides in yet an additional aspect, a method for reducing the elimination of a drug from the inside of a cell which is able to express or which expresses P-gp (MDR1).

The method may comprise conjugating the drug with a carrier described herein, thereby forming a conjugate and providing the cell with the conjugate.

- 30 In an exemplary embodiment of the invention, the cell may be a multiple drug resistant cancer cell.

In a further exemplary embodiment of the invention, the cell may be comprised within an extracranial tumor of an individual.

5 In an additional exemplary embodiment of the invention, the cell may be comprised within a brain of an individual. The cell may be a tumor cell, such as for example a brain tumor cell (primary) or a metastatic brain tumor cell.

10 Means for providing a cell with a conjugate is, for example, to provide the conjugate to an individual who comprises a multiple drug resistant cell or a multiple drug resistant tumor cell (e.g. or a cell which expresses MDR1).

In accordance with the present invention the method may be used to reduce elimination of a drug which is a P-gp substrate or which may be a P-gp substrate.

15 In another aspect, the present invention provides a method for reducing the growth of a cell. The method may comprise contacting the cell with a conjugate which may comprise a) a carrier which may be selected from the group consisting of aprotinin, a biologically active aprotinin fragment, Angiopep-1, Angiopep-2 and biologically active analogs, derivatives and; b) a drug able to reduce the growth of a cell, or with the pharmaceutical composition
20 described herein.

In accordance with the present invention the carrier may be used to increase the potency (efficiency, effectiveness) of the small molecule drug.

25 Also in accordance with the present invention, conjugation of the small molecule drug with the carrier may be achieved through several means which may include using a linker (e.g., a linker able to generate an ester bond) which may allow association of the drug through a suitable atom, for example, through an oxygen atom.

30 In an additional aspect the present invention provides a method for modifying the pharmacokinetics of a compound which may be selected, for example, from the group consisting of a label, a protein, a peptide and a small molecule drug, the method may

comprise the step of conjugating the compound with a carrier described herein thereby forming a conjugate and providing the conjugate to an individual in need.

5 In accordance with the present invention, a ratio of one molecule of the compound for each carrier molecule may be used for the conjugation step.

Further in accordance with the present invention, a ratio of at least two molecules of the compound for each carrier molecule may be used for the conjugation step.

10 Also in accordance with the present invention, a ratio of at least three molecules of the compound for each carrier molecule may be used for the conjugation step.

In a particular embodiment of the invention, the compound may be, for example, a small molecule drug.

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In another particular embodiment of the invention, the compound may be, for example, a label.

20 In accordance with the present invention, the individual in need may be, more particularly, an individual having a tumor. For example, the individual may have a brain tumor. In accordance with the present invention, the brain tumor may be, for example, a primary brain tumor. Also in accordance with the present invention, the brain tumor may be, for example, of a different tissue origin than brain tissue.

25 In an exemplary embodiment of the invention, the brain tumor of the individual may be, for example, an extracranial brain tumor.

In accordance with the present invention, the tumor may comprise a tumor cell expressing or which is able to express P-gp (MDR1).

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The brain tumor may originate, for example, from a lung tumor. The brain tumor may also originate, for example, from a breast tumor. Also, for example, the brain tumor may originate from a melanoma.

In accordance with a particular embodiment of the present invention, the method may increase (optimize), for example, an anti-tumor growth effect of an anticancer drug (as compared with unconjugated drug).

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In accordance with another particular embodiment of the present invention, the method may promote, for example, the accumulation of the compound within a cell (i.e., inside a cell).

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In accordance with yet another particular embodiment of the present invention, the method may allow a reduction in the elimination of the small molecule drug from the inside of a cell (e.g., a cell expressing P-gp).

In accordance with an additional embodiment of the present invention, the method may allow a reduction of cell growth (e.g., reduction of tumor cell growth).

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Furthermore, the method may allow an improvement in the bioavailability of the small molecule drug.

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In addition and in accordance with the present invention, the method may be used to change the (usual) tissue distribution of the small molecule drug.

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In yet a further aspect, the present invention provides for the use of a carrier, conjugate or the pharmaceutical composition as described herein for reducing LRP-dependent accumulation of RAP and for reducing RAP-mediated cellular (e.g., intracellular) event or effect.

For the purpose of the present invention the following terms are defined below.

The term "Angiopep" as used herein refers to Angiopep-1 and Angiopep-2.

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The terms "Taxol-Angiopep" and "Tx1An" are used interchangeably and refer to Taxol-Angiopep-1 and Taxol-Angiopep-2, comprising 1, 2 or 3 Taxol molecules.

The terms "Taxol-Angiopep-1" and "Tx1An1" are used interchangeably and refer to Taxol-Angiopep-1, comprising either 1, 2 or 3 Taxol molecules.

The terms "Taxol-Angiopep-2" and "Tx1An2" are used interchangeably and refer to Taxol-Angiopep-2, comprising either 1, 2 or 3 Taxol molecules.

The term "Tx1An1 (2:1)" refers to the ratio of Taxol over Angiopep-1 molecules in a given conjugate. For example, the term "Tx1An1 (2:1)" relates to a conjugate having 2 molecules of Taxol associated with one molecule of Angiopep-1. In addition, the term "Tx1An1 (3:1)" relates to a conjugate having 3 molecules of Taxol associated with one molecule of Angiopep-1.

Similarly, the term "Tx1An2 (2:1)" refers to the ratio of Taxol over Angiopep-2 molecules in a given conjugate. For example, the term "Tx1An2 (2:1)" relates to a conjugate having 2 molecules of Taxol associated with one molecule of Angiopep-2. In addition, the term "Tx1An2 (3:1)" relates to a conjugate having 3 molecules of Taxol associated with one molecule of Angiopep-2.

The term "carrier" or "vector" is intended to mean a compound or molecule that is able to transport a molecule at a desired targeted site or cell. A carrier may be attached to (covalently or not) or conjugated to another compound or agent and thereby may be able to transport the other compound or agent a desired targeted site. The carrier may be, but is not limited to, a protein, a peptide or to a peptidomimetic and can be naturally occurring or produced by chemical synthesis or recombinant genetic technology (genetic engineering).

The expression "small molecule drug" is intended to mean a drug having a molecular weight of 1000 g/mol or less or between 300 and 700 g/mol.

The terms "treatment", "treating" and the like are intended to mean obtaining a desired pharmacologic and/or physiologic effect, e.g., inhibition of cancer cell growth, death of a cancer cell or amelioration of a neurological disease or condition. The effect may be prophylactic in terms of completely or partially preventing a disease or symptom thereof and/or may be therapeutic in terms of a partial or complete cure for a disease and/or adverse effect attributable to the disease. "Treatment" as used herein covers any treatment of a disease in a mammal, particularly a human, and includes: (a) preventing a disease (e.g., preventing cancer) or condition from occurring in an individual who may be predisposed to the disease but has not yet been diagnosed as having it; (b) inhibiting a disease, (e.g., arresting its development); or (c) relieving a disease (e.g., reducing symptoms associated

with a disease). "Treatment" as used herein covers any administration of a pharmaceutical agent or compound to an individual to treat, cure, alleviate, improve, diminish or inhibit a condition in the individual, including, without limitation, administering a drug comprising a carrier described herein or a conjugate to an individual in need thereof.

- 5 The term "cancer" is intended to mean any cellular malignancy whose unique trait is the loss of normal controls which results in unregulated growth, lack of differentiation and ability to invade local tissues and metastasize. Cancer can develop in any tissue of any organ. More specifically, cancer is intended to include, without limitation, cancer of the brain.

- 10 The term "administering" and "administration" is intended to mean a mode of delivery including, without limitation, intra-arterially, intra-nasally, intra-peritoneally, intravenously, intramuscularly, sub-cutaneously, transdermally or *per os*. A daily dosage may be divided into one, two or more doses in a suitable form to be administered at one, two or more times throughout a time period.

- 15 The term "therapeutically effective" or "effective amount" is intended to mean an amount of a compound sufficient to substantially improve some symptom associated with a disease or a medical condition. For example, in the treatment of cancer, an agent or compound which decreases, prevents, delays, suppresses, or arrests any symptom of the disease or condition would be therapeutically effective. A therapeutically effective amount of an agent or compound is not required to cure a disease or condition but will provide a treatment for a
20 disease or condition such that the onset of the disease or condition is delayed, hindered, or prevented, or the disease or condition symptoms are ameliorated, or the term of the disease or condition is changed or, for example, is less severe or recovery is accelerated in an individual.

- 25 The carrier and conjugate of the present invention may be used in combination with either conventional methods of treatment and/or therapy or may be used separately from conventional methods of treatment and/or therapy.

- When the conjugates of this invention are administered in combination therapies with other agents, they may be administered sequentially or concurrently to an individual. Alternatively, pharmaceutical compositions according to the present invention may be
30 comprised of a combination of a carrier-agent conjugate of the present invention in

association with a pharmaceutically acceptable carrier or pharmaceutically acceptable excipient, as described herein, and another therapeutic or prophylactic agent known in the art.

Pharmaceutically acceptable acid (addition) salts may be prepared by methods known and
5 used in the art.

As used herein, "pharmaceutical composition" means therapeutically effective amounts of the agent together with pharmaceutically acceptable diluents, preservatives, solubilizers, emulsifiers, adjuvant and/or carriers. A "therapeutically effective amount" as used herein refers to that amount which provides a therapeutic effect for a given condition and
10 administration regimen. Such compositions are liquids or lyophilized or otherwise dried formulations and include diluents of various buffer content (e.g., Tris-HCl, acetate, phosphate), pH and ionic strength, additives such as albumin or gelatin to prevent absorption to surfaces, detergents (e.g., Tween 20, Tween 80, Pluronic F68, bile acid salts). Solubilizing agents (e.g., glycerol, polyethylene glycerol), anti-oxidants (e.g., ascorbic acid, sodium
15 metabisulfite), preservatives (e.g., thimerosal, benzyl alcohol, parabens), bulking substances or tonicity modifiers (e.g., lactose, mannitol), covalent attachment of polymers such as polyethylene glycol to the protein, complexation with metal ions, or incorporation of the material into or onto particulate preparations of polymeric compounds such as polylactic acid, polyglycolic acid, hydrogels, etc, or onto liposomes, microemulsions, micelles,
20 unilamellar or multilamellar vesicles, erythrocyte ghosts, or spheroplasts. Such compositions will influence the physical state, solubility, stability, rate of in vivo release, and rate of in vivo clearance. Controlled or sustained release compositions include formulation in lipophilic depots (e.g., fatty acids, waxes, oils). Also comprehended by the invention are particulate compositions coated with polymers (e.g., poloxamers or poloxamines). Other
25 embodiments of the compositions of the invention incorporate particulate forms protective coatings, protease inhibitors or permeation enhancers for various routes of administration, including parenteral, pulmonary, nasal, oral, vaginal, rectal routes. In one embodiment the pharmaceutical composition is administered parenterally, paracancerally, transmucosally, transdermally, intramuscularly, intravenously, intradermally, subcutaneously,
30 intraperitoneally, intraventricularly, intracranially and intratumorally.

Further, as used herein "pharmaceutically acceptable carrier" or "pharmaceutical carrier" are known in the art and include, but are not limited to, 0.01-0.1 M or 0.05 M phosphate buffer or 0.8 % saline. Additionally, such pharmaceutically acceptable carriers may be a buffer, an aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers such as those based on Ringer's dextrose, and the like. Preservatives and other additives may also be present, such as, for example, antimicrobials, antioxidants, collating agents, inert gases and the like.

A "fragment" is to be understood herein as a polypeptide originating from (encompassing) a portion of an original or parent sequence. Fragments encompass polypeptides having truncations of one or more amino acids, wherein the truncation may originate from the amino terminus (N-terminus), carboxy terminus (C-terminus), or from the interior of the protein. A fragment may comprise the same sequence as the corresponding portion of the original sequence. Biologically active fragments of the carrier described herein are encompassed by the present invention.

A "derivative" in the context of proteins or peptides is to be understood herein as a polypeptide originating from an original sequence or from a portion of an original sequence and which may comprise one or more modification; for example, one or more modification in the amino acid sequence (e.g., an amino acid addition, deletion, insertion, substitution etc.) and one or more modification in the backbone or side-chain of one or more amino acid, or an addition of a group or another molecule to one or more amino acids (side-chains or backbone). Biologically active derivatives of the carrier described herein are encompassed by the present invention.

An "analogue" in the context of proteins or peptides is to be understood herein as a molecule having a biological activity and chemical structure similar to that of a polypeptide described herein. An analog comprises a polypeptide which may have, for example, one or more

amino acid insertion, either at one or both of the end of the polypeptide and/or inside the amino acid sequence of the polypeptide.

An "analogue" may have sequence similarity with that of an original sequence or a portion of an original sequence and may also have a modification of its structure as discussed herein.

5 For example, an "analogue" may have at least 90 % sequence similarity with an original sequence or a portion of an original sequence. An "analogue" may also have, for example; at least 70 % or even 50 % sequence similarity with an original sequence or a portion of an original sequence. An "analogue" may have, for example, 50 %, 70%, 80% or 90% sequence similarity to an original sequence with a combination of one or more modification
10 in a backbone or side-chain of an amino acid, or an addition of a group or another molecule, etc. Amino acids which are intended to be similar (a conservative amino acid) to another are known in the art and includes, for example, those listed in Table 1.

In addition, an "analogue" may have at least 50 %, 70%, 80% or 90% sequence identity with
15 an original sequence or a portion of an original sequence. Also, an "analogue" may have, for example, 50 %, 70%, 80% or 90% sequence identity to an original sequence with a combination of one or more modification in a backbone or side-chain of an amino acid, or an addition of a group or another molecule, etc.

20 Similarity or identity may be compared, for example, over a region of 2, 3, 4, 5, 10, 19, 20 amino acids or more (and any number therebetween). Identity may include herein, amino acids which are identical to the original peptide and which may occupy the same or similar position when compared to the original polypeptide. An analog which have, for example, 50% identity with an original polypeptide may include for example, an analog comprising
25 50% of the amino acid of the original polypeptide and similarly with the other percentages. It is to be understood herein that gaps may be found between the amino acids of an analogs which are identical or similar to amino acids of the original peptide. The gaps may include no amino acids, one or more amino acids which are not identical or similar to the original peptide. Biologically active analogs of the carrier of the present invention are encompassed
30 herewith.

Thus, biologically active polypeptides in the form of the original polypeptides, fragments (modified or not), analogues (modified or not), derivatives (modified or not), homologues, (modified or not) of the carrier described herein are encompassed by the present invention.

- 5 Therefore, any polypeptide having a modification compared to an original polypeptide which does not destroy significantly a desired biological activity is encompassed herein. It is well known in the art, that a number of modifications may be made to the polypeptides of the present invention without deleteriously affecting their biological activity. These modifications may, on the other hand, keep or increase the biological activity of the original
- 10 polypeptide or may optimize one or more of the particularity (e.g. stability, bioavailability, etc.) of the polypeptides of the present invention which, in some instance might be needed or desirable. Polypeptides of the present invention comprises for example, those containing amino acid sequences modified either by natural processes, such as posttranslational processing, or by chemical modification techniques which are known in the art.
- 15 Modifications may occur anywhere in a polypeptide including the polypeptide backbone, the amino acid side-chains and the amino- or carboxy-terminus. It will be appreciated that the same type of modification may be present in the same or varying degrees at several sites in a given polypeptide. Also, a given polypeptide may contain many types of modifications. Polypeptides may be branched as a result of ubiquitination, and they may be cyclic, with or
- 20 without branching. Cyclic, branched and branched cyclic polypeptides may result from posttranslational natural processes or may be made by synthetic methods. Modifications comprise for example, without limitation, pegylation, acetylation, acylation, addition of acetomidomethyl (Acm) group, ADP-ribosylation, alkylation, amidation, biotinylation, carbamoylation, carboxyethylation, esterification, covalent attachment to flavin, covalent
- 25 attachment to a heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of drug, covalent attachment of a marker (e.g., fluorescent, radioactive, etc.), covalent attachment of a lipid or lipid derivative, covalent attachment of phosphatidylinositol, cross-linking, cyclization, disulfide bond formation, demethylation, formation of covalent cross-links, formation of cystine, formation of pyroglutamate,
- 30 formylation, gamma-carboxylation, glycosylation, GPI anchor formation, hydroxylation, iodination, methylation, myristoylation, oxidation, proteolytic processing, phosphorylation, prenylation, racemization, selenoylation, sulfation, transfer-RNA mediated addition of amino acids to proteins such as arginylation and ubiquitination, etc. It is to be understood herein

that more than one modification to the polypeptides described herein are encompassed by the present invention to the extent that the biological activity is similar to the original (parent) polypeptide.

- 5 As discussed above, polypeptide modification may comprise, for example, amino acid insertion (i.e., addition), deletion and substitution (i.e., replacement), either conservative or non-conservative (e.g., D-amino acids, desamino acids) in the polypeptide sequence where such changes do not substantially alter the overall biological activity of the polypeptide.
- 10 Example of substitutions may be those, which are conservative (i.e., wherein a residue is replaced by another of the same general type or group) or when wanted, non-conservative (i.e., wherein a residue is replaced by an amino acid of another type). In addition, a non-naturally occurring amino acid may substitute for a naturally occurring amino acid (i.e., non-naturally occurring conservative amino acid substitution or a non-naturally occurring non-
- 15 conservative amino acid substitution).

As is understood, naturally occurring amino acids may be sub-classified as acidic, basic, neutral and polar, or neutral and non-polar. Furthermore, three of the encoded amino acids are aromatic. It may be of use that encoded polypeptides differing from the determined

20 polypeptide of the present invention contain substituted codons for amino acids, which are from the same type or group as that of the amino acid to be replaced. Thus, in some cases, the basic amino acids Lys, Arg and His may be interchangeable; the acidic amino acids Asp and Glu may be interchangeable; the neutral polar amino acids Ser, Thr, Cys, Gln, and Asn may be interchangeable; the non-polar aliphatic amino acids Gly, Ala, Val, Ile, and Leu are

25 interchangeable but because of size Gly and Ala are more closely related and Val, Ile and Leu are more closely related to each other, and the aromatic amino acids Phe, Trp and Tyr may be interchangeable.

It should be further noted that if the polypeptides are made synthetically, substitutions by

30 amino acids, which are not naturally encoded by DNA (non-naturally occurring or unnatural amino acid) may also be made.

A non-naturally occurring amino acid is to be understood herein as an amino acid which is not naturally produced or found in a mammal. A non-naturally occurring amino acid comprises a D-amino acid, an amino acid having an acetylaminoethyl group attached to a sulfur atom of a cysteine, a pegylated amino acid, etc. The inclusion of a non-naturally occurring amino acid in a defined polypeptide sequence will therefore generate a derivative of the original polypeptide. Non-naturally occurring amino acids (residues) include also the omega amino acids of the formula $\text{NH}_2(\text{CH}_2)_n\text{COOH}$ wherein n is 2-6, neutral nonpolar amino acids, such as sarcosine, t-butyl alanine, t-butyl glycine, N-methyl isoleucine, norleucine, etc. Phenylglycine may substitute for Trp, Tyr or Phe; citrulline and methionine sulfoxide are neutral nonpolar, cysteic acid is acidic, and ornithine is basic. Proline may be substituted with hydroxyproline and retain the conformation conferring properties.

It is known in the art that analogues may be generated by substitutional mutagenesis and retain the biological activity of the polypeptides of the present invention. These analogues have at least one amino acid residue in the protein molecule removed and a different residue inserted in its place. Examples of substitutions identified as "conservative substitutions" are shown in Table 1. If such substitutions result in a change not desired, then other type of substitutions, denominated "exemplary substitutions" in Table 1, or as further described herein in reference to amino acid classes, are introduced and the products screened.

In some cases it may be of interest to modify the biological activity of a polypeptide by amino acid substitution, insertion, or deletion. For example, modification of a polypeptide may result in an increase in the polypeptide's biological activity, may modulate its toxicity, may result in changes in bioavailability or in stability, or may modulate its immunological activity or immunological identity. Substantial modifications in function or immunological identity are accomplished by selecting substitutions that differ significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation. (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. Naturally occurring residues are divided into groups based on common side chain properties:

- (1) hydrophobic: norleucine, methionine (Met), Alanine (Ala), Valine (Val), Leucine (Leu), Isoleucine (Ile)

- (2) neutral hydrophilic: Cysteine (Cys), Serine (Ser), Threonine (Thr)
- (3) acidic: Aspartic acid (Asp), Glutamic acid (Glu)
- (4) basic: Asparagine (Asn), Glutamine (Gln), Histidine (His), Lysine (Lys), Arginine (Arg)
- 5 (5) residues that influence chain orientation: Glycine (Gly), Proline (Pro); and aromatic: Tryptophan (Trp), Tyrosine (Tyr), Phenylalanine (Phe)

Non-conservative substitutions will entail exchanging a member of one of these classes for another.

Table 1. amino acid substitution

Original residue	Exemplary substitution	Conservative substitution
Ala (A)	Val, Leu, Ile	Val
Arg (R)	Lys, Gln, Asn	Lys
Asn (N)	Gln, His, Lys, Arg	Gln
Asp (D)	Glu	Glu
Cys (C)	Ser	Ser
Gln (Q)	Asn	Asn
Glu (E)	Asp	Asp
Gly (G)	Pro	Pro
His (H)	Asn, Gln, Lys, Arg	Arg
Ile (I)	Leu, Val, Met, Ala, Phe, norleucine	Leu
Leu (L)	Norleucine, Ile, Val, Met, Ala, Phe	Ile
Lys (K)	Arg, Gln, Asn	Arg
Met (M)	Leu, Phe, Ile	Leu
Phe (F)	Leu, Val, Ile, Ala	Leu
Pro (P)	Gly	Gly
Ser (S)	Thr	Thr
Thr (T)	Ser	Ser
Trp (W)	Tyr	Tyr
Tyr (Y)	Trp, Phe, Thr, Ser	Phe
Val (V)	Ile, Leu, Met, Phe, Ala, norleucine	Leu

A biologically active analog may be, for example, an analogue having at least one conservative amino acid substitution in the original sequence. A biologically active analog may also be for example, an analog having an insertion of an amino acid.

5

For example, an Angiopep-1 analog may have the formula I : X_1 -Angiopep-1- X_2

For example, an Angiopep-2 analog may have the formula II : X_1 -Angiopep-2- X_2

X_1 and X_2 may independently be an amino acid sequence of from between 0 to about 100 (e.g., from between 0 to about 60) amino acids. X_1 and X_2 may be derived from consecutive amino acids of aprotinin or aprotinin analogs (homologous amino acid sequence) or may be any other amino acid sequence (heterologous amino acid sequence). A compound of either formula I or II, may also comprises an amino acid substitution, deletion or insertion within the amino acid sequence of Angiopep-1 or Angiopep-2. The analog however would preferably be biologically active as determined by one of the assays described herein or by any similar or equivalent assays.

Examples of aprotinin analogs may be found by performing a protein blast (Genebank: www.ncbi.nlm.nih.gov/BLAST/) of the synthetic aprotinin sequence (or portion thereof) disclosed in international application no. PCT/CA2004/000011. Exemplary aprotinin analogs may be found, for example under accession nos. CAA37967 (GI:58005), 1405218C (GI:3604747) etc.

A biologically active fragment of a polypeptide (e.g., of 19 amino acids) described herein may include for example a polypeptide of from about 7, 8, 9 or 10 to 18 amino acids.

A biologically active polypeptide (e.g., carrier) may be identified by using one of the assays or methods described herein. For example a candidate carrier may be produced by conventional peptide synthesis, conjugated with Taxol as described herein and tested in an *in vivo* model as described in Example 5. A biologically active carrier may be identified, for example, based on its efficacy at reducing tumor burden compared to placebo-treated mice. A small molecule drug candidate which may be used in the conjugation of a carrier described herein may be identified, for example, by determining or not whether the drug is

expulsed from P-gp over-expressing cells as described herein and evaluating whether its conjugation to the carrier increases its accumulation inside a desired cell.

5 Examples of biologically active carrier (i.e., biologically active analog of Angiopep-1 and/or Angiopep-2) may include for example, peptides derived from the kunitz-domain such as TFFYGGCRGKRNNFKTKEY, RFKYGGCLGNKNNYLRLKY and TFFYGGCRAKRNNFKRAKY.

10 Other examples of biologically active analogs may be found in Table 2 and in the Sequence Listing.

Table 2: Design of 96 peptides from similar domain to aprotinin and Angiopep-1 with different charges and amino acid insertions

96 PEPTIDES ORDERED AT SYNPEP (California, USA)																									
Proteins	Characteristics	#Pep	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	
Aprot-synth		1	T	F	V	Y	G	G	C	R	A	K	R	N	N	F	K	R	A	E	K	E	Y		
Bikunin HI-30		2	T	F	F	Q	Y	G	G	C	R	A	K	R	N	N	F	V	T	A	E	K	E	Y	
Amyloid		3	P	F	F	Y	G	G	C	R	A	K	R	N	N	F	D	T	A	E	K	E	Y		
Kunitz-Inhib 1		4	S	F	F	Y	G	G	C	R	A	K	R	N	N	F	K	R	A	E	K	E	Y		
Peptides	CHARGE (+6)	5	T	F	F	F	Y	G	G	C	R	A	K	R	N	N	F	K	R	A	E	K	E	Y	
		6	T	F	F	F	Y	G	G	C	R	A	K	R	N	N	F	K	R	A	E	K	E	Y	
		7	T	F	F	F	Y	G	G	C	R	A	K	R	N	N	F	K	R	A	E	K	E	Y	
		8	T	F	F	F	Y	G	G	C	R	A	K	R	N	N	F	K	R	A	E	K	E	Y	
		9	T	F	F	Q	Y	G	G	C	R	A	K	R	N	N	F	K	R	A	E	K	E	Y	
		10	T	F	F	Q	Y	G	G	C	R	A	K	R	N	N	F	K	R	A	E	K	E	Y	
	CHARGE (+5)	11	T	F	F	F	Y	G	G	C	L	G	K	R	N	N	F	K	R	A	E	K	E	Y	
		12	T	F	F	F	Y	G	G	C	L	G	K	R	N	N	F	K	R	A	E	K	E	Y	
		13	P	F	F	F	Y	G	G	C	R	A	K	R	N	N	F	K	R	A	E	K	E	Y	
		14	T	F	F	F	Y	G	G	C	R	A	K	R	N	N	F	K	R	A	E	K	E	Y	
		15	P	F	F	F	Y	G	G	C	R	A	K	R	N	N	F	K	R	A	E	K	E	Y	
		16	T	F	F	F	Y	G	G	C	R	A	K	R	N	N	F	K	R	A	E	K	E	Y	
		17	P	F	F	F	Y	G	G	C	R	A	K	R	N	N	F	K	R	A	E	K	E	Y	
		18	T	F	F	F	Y	G	G	C	R	A	K	R	N	N	F	K	R	A	E	K	E	Y	
	CHARGE (+4)	19	T	F	F	F	Y	G	G	C	R	A	K	R	N	N	F	D	R	A	E	K	E	Y	
		20	T	F	F	F	Y	G	G	C	R	A	K	R	N	N	F	K	R	A	E	K	E	Y	
		21	P	F	F	F	Y	G	G	C	R	A	K	R	N	N	F	K	R	A	E	K	E	Y	
		22	T	F	F	F	Y	G	G	C	R	A	K	R	N	N	F	K	R	A	E	K	E	Y	
		23	T	F	F	F	Y	G	G	C	R	A	K	R	N	N	F	K	R	A	E	K	E	Y	
		24	T	F	F	F	Y	G	G	C	R	A	K	R	N	N	F	K	R	A	E	K	E	Y	
		25	T	F	F	F	Y	G	G	S	R	G	N	R	N	N	F	K	T	A	E	K	E	Y	
	CHARGE (+3)	26	T	F	F	F	Y	G	G	C	L	G	N	G	N	N	F	K	R	A	E	K	E	Y	
		27	T	F	F	F	Y	G	G	C	L	G	N	R	N	N	F	K	R	A	E	K	E	Y	
		28	T	F	F	F	Y	G	G	C	L	G	N	R	N	N	F	K	R	A	E	K	E	Y	
		29	T	F	F	F	Y	G	G	C	R	A	K	R	N	N	F	K	R	A	E	K	E	Y	
		30	T	F	F	F	Y	G	G	C	R	A	K	R	N	N	F	D	R	A	E	K	E	Y	
		31	T	F	F	F	Y	G	G	C	R	A	K	R	N	N	F	L	R	A	E	K	E	Y	
		32	T	F	F	F	Y	G	G	C	R	A	K	R	N	N	F	D	R	A	E	K	E	Y	
		33	T	F	F	F	Y	G	G	S	R	G	N	G	N	N	F	D	R	A	E	K	E	Y	
	CHARGE (+2)	34	T	F	F	F	Y	G	G	C	R	A	K	R	N	N	F	V	T	A	E	K	E	Y	
		35	P	F	F	F	Y	G	G	C	R	A	K	R	N	N	F	V	T	A	E	K	E	Y	
		36	T	F	F	F	Y	G	G	C	L	G	N	G	N	N	F	L	T	A	E	K	E	Y	
		37	S	F	F	F	Y	G	G	C	L	G	N	K	N	N	F	V	T	A	E	K	E	Y	
	HUMAN	38	T	F	F	F	Y	G	G	C	L	G	N	K	N	N	F	V	R	A	E	K	E	Y	
	HUMAN	39	T	F	F	F	Y	G	G	C	L	G	N	K	N	N	F	V	R	A	E	K	E	Y	
	HUMAN	40	T	F	F	F	Y	G	G	S	M	G	N	K	N	N	F	V	R	A	E	K	E	Y	
	HUMAN	41	P	F	F	F	Y	G	G	C	L	G	N	R	N	N	F	V	R	A	E	K	E	Y	
	HUMAN	42	T	F	F	F	Y	G	G	C	L	G	N	R	N	N	F	V	R	A	E	K	E	Y	
	HUMAN	43	T	F	F	F	Y	G	G	C	L	G	N	K	N	N	F	V	R	A	E	K	E	Y	
	CHARGE (+1)	44	T	F	F	F	Y	G	G	C	G	G	N	G	N	N	F	L	T	A	E	K	E	Y	
		45	T	F	F	F	Y	G	G	C	R	G	N	R	N	N	F	L	T	A	E	K	E	Y	
		46	T	F	F	F	Y	G	G	C	R	G	N	R	N	N	F	K	T	A	E	K	E	Y	
		47	P	F	F	F	Y	G	G	C	L	G	N	K	N	N	F	K	T	A	E	K	E	Y	
		48	T	F	F	F	Y	G	G	C	R	A	K	R	N	N	F	K	T	A	E	K	E	Y	
		49	T	F	F	F	Y	G	G	C	R	A	K	R	N	N	F	K	T	A	E	K	E	Y	
	HUMAN	50	P	F	F	F	Y	G	G	C	G	G	N	G	N	N	F	V	R	A	E	K	E	Y	
	HUMAN	51	S	F	F	F	Y	G	G	C	M	G	N	G	N	N	F	V	R	A	E	K	E	Y	
	HUMAN	52	P	F	F	F	Y	G	G	C	G	G	N	G	N	N	F	V	R	A	E	K	E	Y	
	HUMAN	53	T	F	F	F	Y	G	G	C	L	G	N	G	N	N	F	V	R	A	E	K	E	Y	
	HUMAN	54	S	F	F	F	Y	G	G	C	L	G	N	G	N	N	F	V	R	A	E	K	E	Y	
	HUMAN	55	T	F	F	F	Y	G	G	S	L	G	N	G	N	N	F	V	R	A	E	K	E	Y	
	CHARGE (+0)	56	T	F	F	F	Y	G	G	C	R	A	K	R	N	N	F	V	T	A	E	K	E	Y	
		57	T	F	F	F	Y	G	G	C	L	G	N	R	N	N	F	V	S	A	E	K	E	Y	
		58	T	F	F	F	Y	G	G	C	L	G	N	R	N	N	F	D	R	A	E	K	E	Y	
	HUMAN	59	T	F	F	F	Y	G	G	C	L	G	N	R	N	N	F	L	R	A	E	K	E	Y	
	HUMAN	60	T	F	F	F	Y	G	G	C	L	G	N	K	N	N	F	L	R	A	E	K	E	Y	
	HUMAN	61	P	F	F	F	Y	G	G	C	G	G	N	R	N	N	F	L	R	A	E	K	E	Y	
	HUMAN	62	P	F	F	F	Y	G	G	S	G	G	N	R	N	N	F	L	R	A	E	K	E	Y	
Aprotinin	vs APROTININ N-term	63	H	R	P	D	F	C	L	E	P	P	Y	T	G	P	C	V	A	R	I	Y	G	Y	
	(1 helix, A-term)	64	A	R	I	I	R	C	R	A	K	R	N	N	Y	K	A	S	A	E	D	C	I	R	
	(2 β sheets, Y-term)	65	Y	G	G	C	R	A	K	R	N	N	A	Y	K	A	S	A	E	D	C	I	R	Y	
	(1 α, 1 β)	66	P	D	F	C	L	E	P	P	Y	T	G	P	C	V	A	R	I	I	R	Y	G	Y	
AngioPep	AngioPep-1	67	T	F	F	F	Y	G	G	C	R	A	K	R	N	N	F	K	T	A	E	K	E	Y	
	AngioPep1 (lysine)	68	K	F	F	F	Y	G	G	C	R	A	K	R	N	N	F	K	T	A	E	K	E	Y	
	AngioPep1 (4Y)	69	T	F	F	F	Y	G	G	C	R	A	K	R	N	N	F	K	T	A	E	K	E	Y	
	cys bridge	70	T	F	F	F	Y	G	G	C	R	A	K	R	N	N	F	K	T	A	E	K	E	Y	
	cys-Nterminal	71	C	T	F	F	Y	G	G	C	R	A	K	R	N	N	F	K	T	A	E	K	E	Y	
	cys-Cterminal	72	T	F	F	F	Y	G	G	C	R	A	K	R	N	N	F	K	T	A	E	K	E	Y	
	cys-Nterminal	73	C	T	F	F	Y	G	G	S	C	R	A	K	R	N	N	F	K	T	A	E	K	E	Y
	cys-Cterminal	74	T	F	F	F	Y	G	G	C	R	A	K	R	N	N	F	K	T	A	E	K	E	Y	
	pro	75	P	F	F	F	Y	G	G	C	R	A	K	R	N	N	F	K	T	A	E	K	E	Y	
	charge (+3)	76	T	F	F	F	Y	G	G	C	R	A	K	R	N	N	F	K	T	A	E	K	E	Y	
	charge (+3)-cys	77	T	F	F	F	Y	G	G	K	R	A	K	R	N	N	F	K	T	A	E	K	E	Y	
	charge (+4)	78	T	F	F	F	Y	G	G	C	R	A	K	R	N	N	F	K	T	A	E	K	E	Y	
	charge (+4)-cys	79	T	F	F	F	Y	G	G	K	R	A	K	R	N	N	F	K	T	A	E	K	E	Y	
	charge (+5)	80	T	F	F	F	Y	G	G	K	R	A	K	R	N	N	F	K	T						

It is to be understood herein, that if a "range" or "group of substances" is mentioned with respect to a particular characteristic (e.g., temperature, concentration, time and the like) of the present invention, the present invention relates to and explicitly incorporates herein each and every specific member and combination of sub-ranges or sub-groups therein whatsoever.

5 Thus, any specified range or group is to be understood as a shorthand way of referring to each and every member of a range or group individually as well as each and every possible sub-ranges or sub-groups encompassed therein; and similarly with respect to any sub-ranges or sub-groups therein. Thus, for example,

- 10 - with respect to a length of 19 amino acid long or less, is to be understood as specifically incorporating herein each and every individual length, e.g., a length of 18, 17, 15, 10, 5, etc.; Therefore, unless specifically mentioned, every range mentioned herein is to be understood as being inclusive. For example, in the expression from 5 to 19 amino acids long is to be as including 5 and 19;
- 15 - and similarly with respect to other parameters such as sequences, length, concentrations, elements, etc...

It is in particular to be understood herein that the sequences, regions, portions defined herein each include each and every individual sequences, regions, portions described thereby as well as each and every possible sub-sequences, sub-regions, sub-portions whether such sub-sequences, sub-regions, sub-portions is defined as positively including particular
20 possibilities, as excluding particular possibilities or a combination thereof; for example an exclusionary definition for a region may read as follows: "provided that said polypeptide is no shorter than 4, 5, 6, 7, 8 or 9 amino acids. Yet a further example of a negative limitation is the following; a sequence comprising SEQ ID NO.: X with the exclusion of a polypeptide of SEQ ID NO. Y.; etc. Other examples of exemplary negative limitations are the following;
25 "other than brain cancer" or "other than brain tissue" or "other than brain cells".

BRIEF DESCRIPTION OF THE DRAWINGS

30 In drawings which illustrates exemplary embodiments of the invention,

Fig. 1. is an amino sequence of aprotinin-derived peptides;

Fig. 2. is a schematic representation of the efflux pump, P-glycoprotein (P-gp or MDR1) at the cell surface. The efflux pump, P-gp or MDR1, associated with multidrug resistance is highly expressed at the cell surface of many cancer cells and various tissues including the blood-brain barrier;

5

Fig. 3. is a schematic representation of conjugation of a drug to the carrier peptide of the present invention;

10

Fig. 4. is a chromatogram illustrating the production in high amount of Tx1An2 (3:1) conjugate;

Fig. 5. is a HPLC analysis of the peak purified on a hydrophobic column using AKTA-explorer;

15

Fig. 6. is a diagram illustrating the *In situ* brain perfusion of radiolabeled Angiopep-2 and the vascular marker inulin;

Fig. 7. is an histogram illustrating the apparent distribution volume of transferrin, aprotinin and Angiopeps in total brain, brain capillaries and brain parenchyma;

20

Fig. 8. is a diagram of cell proliferation in the presence of the parent drug Taxol. Glioblastoma cells (U-87) were exposed to various concentrations of Taxol for 3-days. ³H-Thymidine incorporated in cells were plotted as a function of Taxol concentrations;

25

Fig. 9A is a diagram representing the accumulation of various drugs in MDCK cells transfected with *MDR1* in the presence or absence (control) of 10uM CsA, a P-gp inhibitor. The experiment was performed in the presence of 1% DMSO;

Fig. 9B. is a diagram representing the accumulation of the conjugate in cells over-expressing P-gp.

30

Fig. 10A and B are diagrams representing tissue distribution of Taxol and Tx1An1 conjugate,

Fig. 11 is a diagram representing lung distribution of Taxol and Tx1An1,

Fig. 12 is a diagram representing the levels of Tx1An1 conjugate in plasma and lung,

5 **Fig. 13** is a diagram representing the effect of Tx1An2 treatment on subcutaneous glioblastomas (U-87) tumor growth;

Fig. 14. are photographs illustrating the detection of β -tubulin in NCI-H460 by immunofluorescence or visible light in cancer cells exposed to Taxol or Tx1An2 conjugate, as
10 control, cells were exposed to to 1% DMSO;

Fig. 15. are diagrams illustrating the effect of Taxol and Tx1An2 conjugate on NCI-H460 cell-cycle measured by FACS. Cells were exposed for 24hrs with the vehicle (DMSO), Taxol (100 nM) or Tx1An2 conjugate (30 nM, equivalent to 100 nM of Taxol);

15

Fig. 16A and 16B are pictures representing immunodetection of LRP in human brain tumor biopsies;

Fig. 17A. is a diagram illustrating the accumulation of [125 I]-RAP in fibroblasts MEF-1 and
20 PEA-13 in the presence of various aprotinin concentrations.

Fig. 17B. is a diagram expressing the results of **Fig. 17A**, where the LRP-dependent accumulation of [125 I]-RAP was calculated by subtracting the results for the uptake obtained with PEA-13 cells from the results with MEF-1 and expressed as a function of aprotinin
25 concentrations;

Fig. 18. is an histogram illustrating the effect of aprotinin and Angiopep-2 on RAP uptake. The accumulation of [125 I]-RAP in fibroblasts MEF-1 and PEA-13 was measured in the presence of 25 μ M aprotinin or Angiopep. The LRP-dependent accumulation of [125 I]-RAP
30 was calculated by subtracted the results for the uptake obtained with PEA-13 cells from the results obtained with MEF-1;

Fig. 19A is a diagram illustrating the blood kinetics of Tx1An2 (3:1) conjugate in DMSO 80% after a bolus injection;

Fig. 19B is a diagram illustrating the blood kinetics of Tx1An2 (3:1) conjugate in Solutol[®] HS15 20% after a bolus injection;

Fig. 19C is a diagram illustrating the blood kinetics of Taxol in DMSO 80% after a bolus injection;

Fig. 20 are diagrams of tissue distribution of Tx1An2 (3:1) conjugate diluted in DMSO (80%) or Solutol[®] HS15 (20%);

Fig. 21A is an histogram illustrating tissue distribution of Taxol, Tx1An2 (3:1) and Tx1An2 (2:1) after intraveinuous (i.v.) injection in several tissues;

Fig. 21B is an histogram illustrating tissue distribution of Taxol, Tx1An2 (3:1) and Tx1An2 (2:1) after intraveinuous (i.v.) injection in normal and tumoral brain;

Fig. 22A is a graph illustrating tumor volume following i.v. administration of Taxol (10 mg/kg) or Tx1An2 (3:1) conjugate (20 mg/kg) formulation (in Solutol[®]) in mice with NCI-H460 cells implanted in their right flank;

Fig. 22B is a graph illustrating tumor volume following i.p. administration of Tx1An2 (2:1) or Tx1An2 (3:1) conjugate formulation (Solutol[®]) in mice with NCI-H460 cells implanted in their right flank;

Fig. 23A is a graph illustrating tumor volume following administration of either the vehicle (control), Taxol (10 mg/kg) or Tx1An2 (3:1) formulation (20 mg/kg) by i.p. injections or infusion with Alzet pumps (30mg/kg/14days) in mice with NCI-H460 cells implanted in their right flank; Treatments are indicated by arrows, and;

Fig. 23B is a graph illustrating tumor volume following administration of either the vehicle (control), Taxol (10 mg/kg) or Tx1An2 (3:1) formulation (20 mg/kg) by i.p. injections or

infusion with Alzet pumps in mice with U87 cells implanted in their right flank; Treatments are indicated by arrows.

5 DETAILED DESCRIPTION OF THE INVENTION

Angiopep-1 and -2 represents two non-limitative, exemplary embodiments of aprotinin derived peptides which have been tested herein (Fig.1). Taxol which represents a non-limitative exemplary embodiment of a molecule or compound conjugated to the carrier of the present invention was chosen as a candidate anticancer drug as this natural compound, isolated from the bark and needles of the yew tree, is a highly efficient chemotherapeutic. Moreover, this compound is approved by the Food and Drug Administration (FDA) for ovarian cancer, breast cancer, non-small cell lung cancer and Kaposi's sarcoma and is a well characterized anticancer agent.

15 EXAMPLES

Cell proliferation assay

For the *in vitro* cell proliferation assay, between 2.5 and 5 X 10⁴ of U87 or A549 cells were seeded in a 24 well tissue culture microplate in a final volume of 1 ml of medium with 10% serum and incubated for 24 hours at 37°C and 5% CO₂. The medium was then replaced with serum-free medium and incubated overnight. The next morning the drug was freshly dissolved in dimethyl sulfoxide (DMSO) and the medium was replaced with complete medium containing the drug at different concentrations in triplicates. The final concentration of DMSO was 0.1%. The control used is a microplate well with cells and without drug. The cells were incubated for 48 to 72 hrs at 37°C and 5% CO₂. After the incubation, the medium was changed and replaced with 1 ml of complete medium containing [³H]-thymidine (1 µCi/assay). The plate was incubated at 37°C and 5% CO₂ for 4 hrs. The medium was removed and the cells washed with PBS heated at 37°C. The cells were fixed with a mix of ethanol:acetic acid (3;1), then washed with water and precipitated 3 times with 10% of ice-cold TCA (trichloroacetic acid). Finally 500 µl of PCA (perchloric acid) were added to the wells and the microplates were heated for 30 min at 65 °C and 30 min at 75°C. The contents of each well was then transferred in a scintillation vial with 10 ml of scintillation cocktail and the activity was measured in CPM (count per minute) on a liquid scintillation counter Tri-Carb from Packard.

Iodination of peptides

Peptides were iodinated with standard procedures using iodo-beads from Sigma. Both Angiopep-1 and Angiopep-2 were diluted in 0.1M phosphate buffer, pH 6.5 (PB). Two iodo-beads were used for each protein. These beads were washed twice with 3 ml of PB on a whatman filter and re-suspended in 60 µl of PB. ^{125}I (1 mCi) from Amersham-Pharmacia biotech was added to the bead suspension for 5 min at room temperature. Each iodination was initiated by the addition of the peptide (100 µg). After an incubation of 10 min at room temperature, the free iodine was removed by HPLC.

Subcutaneous implantation

In order to estimate the efficiency of the Taxol-conjugates and formulations on tumor growth, we developed a subcutaneous model of glioblastomas. In this model, 2.5×10^6 cells in 100 µl of cell medium without serum containing 1% methylcellulose were subcutaneously injected in the mice flank. The tumor was clearly visible and could be measured using a vernier caliper. The estimated tumor volume was then plotted as a function of time.

In situ mouse brain perfusion

The uptake of [^{125}I]-peptides to the luminal side of mouse brain capillaries was measured using the in situ brain perfusion method adapted in our laboratory for the study of drug uptake in the mouse brain. Briefly, the right common carotid of ketamine/xylazine (140/8 mg/kg i.p.) anesthetized mice was exposed and ligated at the level of the bifurcation of the common carotid, rostral to the occipital artery. The common carotid was then catheterized rostrally with polyethylene tubing filled with heparin (25 U/ml) and mounted on a 26-gauge needle. The syringe containing the perfusion fluid ([^{125}I]-peptides or [^{14}C]-inulin in Krebs/bicarbonate buffer at a pH 7.4 gassed with 95% O_2 and 5% CO_2) was placed in an infusion pump (Harvard pump PHD 2000; Harvard Apparatus) and connected to the catheter. Prior to the perfusion, the contralateral blood flow contribution was eliminated by severing heart ventricles. The brain was perfused for the indicated times at a flow rate of 1.15 ml/min. After 14.5 min of perfusion, the brain was further perfused for 60 s with Krebs buffer, to wash the excess of [^{125}I]-proteins. Mice were then decapitated to terminate perfusion and the right hemisphere was isolated on ice before being subjected to capillary depletion. Aliquots of homogenates, supernatants, pellets and perfusates were taken to

measure their contents in [¹²⁵I]-conjugates by TCA precipitation and to evaluate the apparent volume of distribution.

5 EXAMPLE 1

Preparation of conjugates

Since the resistance towards various drugs such as vincristine, etoposide, and doxorubicin is mediated through P-gp (MDR1) overexpression (Fig. 2), any methods of bypassing this efflux pump may potentiate the action of these drugs on various cancer types. The bypass of
10 P-gp may therefore be useful to increase the potency of drugs which are associated with resistance mediated by P-gp. Carriers described herein were therefore tested for their ability to bypass P-gp.

The conjugation of a drug to the carrier described herein is illustrated in Fig. 3. Briefly, in
15 order to conjugate Taxol to the Angiopep-1 or Angiopep-2 carrier, Taxol was first activated into N-succinimide (2'-NHS-Txl) derivative. Then amines found for example in lysine residue or amino-terminal of Angiopep-1 or Angiopep-2 reacted on this activated-Taxol by forming a peptide bond (amide bond). In Angiopep-1 or Angiopep-2, the amino-terminal (in position 1) and the lysines (at position 10 and 15) were able to react with activated-Taxol.
20 Therefore, multiple combinations of conjugates was found to occur by the addition of 1, 2 or 3 Taxols to the peptide depending on the molar ratio used (Fig. 3). The whole conjugation was analyzed by HPLC and conjugation was confirmed by Mass spectra (Maldi-Tof). Taxol was found to be releasable from the carrier by the cleavage of the ester bond by esterases. Conjugates were therefore efficiently produced by combining the carrier with an anticancer
25 drug.

In an exemplary embodiment of the present invention, the production of the TxlAn2 (3:1) conjugate, was carried out by directly adding 1 mole equivalent of Angiopep-2 to a solution of 2.5 moles equivalent of 2'-NHS-Taxol. The reaction was performed in 68% DMSO in
30 Ringer solution (pH 7.3), for 1 hr at 12°C and after removing the cold bath, for about 22 hrs at room temperature (Fig. 4). Angiopep-2, 2'-NHS-Taxol and TxlAn2 (3:1) conjugate are shown on the chromatogram by arrows. Aliquots of the reaction were sampled and analyzed by HPLC after 25 min, 2 hrs 15 min, 5 hrs and 23 hrs as indicated in Fig. 4. The peaks of

Angiopep-2, 2'-NHS-Taxol and Tx1An2 (3:1) conjugate are shown by arrow on the chromatogram. Results of Fig. 4 illustrate the disappearance of Angiopep-2 and 2'-NHS-Taxol during the reaction mainly to the profit of the Tx1An2 (3:1) conjugate.

- 5 This mixture of products was separated by hydrophobic chromatography on a RPC 300 mm column with a flow rate at 4 ml/min using AKTA-explorer (Fig. 5). For the peak that corresponds to the Tx1An2 (3:1) conjugate, fractions were pooled, analyzed by HPLC and MS. In Fig. 5, the upper chromatogram corresponds to the running reaction at t=23 hrs whereas the lower one corresponds to the Tx1An2 (3:1) conjugate which has been confirmed
10 by mass spectrometry (MW 5107) after AKTA purification.

EXAMPLE 2

In situ brain perfusion of Taxol-Angiopep-2 conjugates.

- 15 To evaluate the brain uptake of Angiopep *in vivo*, the initial rate of transport for [125 I]-Angiopep into mouse brain parenchyma was measured using *in situ* brain perfusion as described herein. Mouse brain was perfused for the indicated times with either [125 I]-Angiopep-2 or [14 C]-inulin. After perfusion, the brain was further perfused for 60 sec with Ringer solution to wash the excess of radiolabeled molecules and then the right hemisphere
20 was isolated on ice before being subjected to capillary depletion. Aliquots of homogenates, supernatants, pellets and perfusates were taken to measure their contents in [125 I]-Angiopep-2 or [14 C]-inulin. Results obtained for the accumulation for these molecules into the brain parenchyma are illustrated in Fig. 6. The accumulation of [125 I]-Angiopep-2 increased as a function of time and is higher than that of the vascular marker, [14 C]-inulin.

- 25 We further compared the initial brain uptake after a 5 min perfusion for [125 I]-aprotinin, [125 I]-transferrin and [125 I]-Angiopeps (Fig. 7) (1:1). Results show that Angiopep and aprotinin have a highest initial transport rate than transferrin.

30

EXAMPLE 3

Effect of conjugates on cell growth

In an *in vitro* assay, Taxol (unconjugated) was shown herein to block the proliferation of

glioblastoma cells (U-87) with IC₅₀ value of around 10 nM (Fig.8). The effect of Taxol conjugated with the carrier described herein on the proliferation of various cell lines was then evaluated and compared to unconjugated Taxol (referred as Taxol). As shown in Table 3, the IC₅₀ values obtained for the Taxol-Angiopep-2 (Tx1An2) conjugate were very similar to that of unconjugated Taxol in many cancer cells. Endothelial cells from rat brain (RBE4) were less sensitive than the tested cancer cell lines. Overall, these results indicate that the potency of conjugates to block cell proliferation *in vitro* is similar to the unconjugated Taxol. For comparison purposes, results obtained were expressed in term of Taxol concentration.

Table 3. Effect of conjugate on cell proliferation.

Cell lines	IC ₅₀ (nM)	
	Taxol	Taxol-Angiopep-2 (3:1)
Glioblastomas		
U-87	9.5	9.7
U-118	7.2	8.1
Lung carcinoma		
NCI-H460	9.3	12.5
A549	3.6	6.0
Calu-3	17.2	25.0
Endothelial cells		
RBE4	137	139

Most of these cells (U-87, U-118, NCI-H460, A549) express LRP. This data is however unavailable for RBE4 cells.

EXAMPLE 4

By-passing of P-gp by conjugates

In order to determine whether the conjugates of the present invention were P-gp substrates or not, MDCK cells were stably transfected with human *MDR1* (MDCK-MDR1) and were subsequently incubated with unconjugated-anticancer drug or with the conjugates of the present invention. In a first experiment, MDCK-MDR1 cells were incubated with ³H-vinblastine (³H-VBL), rhodamine, ³H-Taxol, ¹²⁵I-Taxol-Angiopep-1 (¹²⁵I-Tx1An1), ¹²⁵I-Taxol-Angiopep-2 (¹²⁵I-Tx1An2) for 1 hr at 37°C in the presence or absence of 10 μM of cyclosporine A (CsA); a P-gp competitive inhibitor (Fig. 9A). After the incubation, cells were washed and accumulation of radioactivity inside the cells or intracellular fluorescence

was quantified. The increased in the accumulation of these drugs is expressed in term of x-fold compared to their respective control measured in the absence of CsA. Thus, the control value for each drug was set to 1-fold.

5 In another experiment, the ability of the conjugates to accumulate in cells overexpressing P-gp was monitored (Fig. 9B). MDCK-*MDR1* cells were incubated with 50 nM of either ^3H -Taxol, ^{125}I -Taxol-Angiopep-1 (^{125}I -Tx1An1) or ^{125}I -Taxol-Angiopep-2 (^{125}I -Tx1An2) for 2 hrs at 37°C. After the incubation, the cells were washed and the radioactivity accumulated in cells was quantified. The results were expressed as drug accumulation in pmole/120min.

10 As shown in Fig. 9A, the accumulation of [^3H]-Taxol increased by 15-fold in the presence of the P-gp competitive inhibitor; cyclosporin A (CsA). The accumulation of rhodamine and [^3H]-vinblastine also increased by 7.5-fold and 10-fold respectively in the presence of CsA. These results show that Taxol, rhodamine and vinblastine are P-gp substrates. However, the
15 lack of CsA effect on the accumulation of both [^{125}I]-Taxol-Angiopep-1 and [^{125}I]-Taxol-Angiopep-2 conjugates, indicates that they are not P-gp substrates. The accumulation of both conjugates, in the absence of CsA, was at least 11-fold higher than of [^3H]-Taxol (Fig. 9B). These later results strongly confirm that both conjugates bypass the action of P-gp since P-gp is not able to expulse them from the cells. These results additionally demonstrate that the
20 presence of a carrier in conjugation with an anticancer drug increases the potency of the drug. Therefore, the carriers described herein are useful for the transport and/or accumulation of drugs inside a cell and are especially useful for drugs which are usually expelled by P-gp (i.e., drugs which are P-gp substrates).

25 EXAMPLE 5

Distribution and pharmacokinetics of conjugates

The impact of conjugation of the drug to the carrier on drug distribution was evaluated by administering either ^3H -Taxol (5 mg/kg) or ^{125}I -Taxol-Angiopep-1 (Tx1An-1) (10 mg/kg,
30 equivalent to 5 mg of Taxol/kg) to mice. The unconjugated anticancer drug and the conjugates were injected intra-veinously (*i.v.*) in mice as a bolus. Tissues were collected at different times (0.25, 0.5, 1 and 4 hrs) and homogenized. In order to quantify the amount of ^3H -Taxol, tissue homogenates were digested with tissue solubilizer (soluble) and 10 ml of

liquid scintillator was then added to samples. The amount of the ^{125}I -labeled conjugate, in the different tissues was measured after TCA precipitation. Radioactivity associated with the tissues was therefore quantified. The area under the curve (AUC₀₋₄) was estimated using the Prism software and was plotted for the different tissues (Fig. 10). Results of Fig. 10A indicate that the AUC₀₋₄ values obtained for the conjugate are higher than that of Taxol in various tissues including the brain, kidney, liver and the eyes which indicates a higher accumulation of the conjugate in these tissues compared to the unconjugated drug. More particularly, results presented in Fig. 10B indicate that the accumulation of the conjugate is much higher than unconjugated drug in the lung.

Results of a similar experimentation conducted with the Taxol-angiopep-2 conjugate are summarized in Table 4 below. Although there is difference with results obtained for the TxlAn-1 conjugate, the conjugate of Table 4 also accumulates in the lungs, brain and liver more efficiently than unconjugated Taxol.

Table 4.

Tissue	AUC 0-4 ($\mu\text{g/g}$ of tissue)		
	TxlAn-2	Taxol	Ratio (TxlAn-2/Taxol)
Plasma	170	2.2	77.3
Brain	0.32	0.07	4.6
Lung	3.4	1.1	3.0
Kidney	11.2	8.0	1.4
Heart	5.0	2.5	2.0
Liver	513	22	23
Eye	0.99	0.57	1.7
Urine	35.7	88	0.4

Treatments equivalent to 5mg/kg of Taxol

The kinetics of Taxol and Taxol-Angiopep-1 accumulation in the lung is also presented in Fig. 11. Results clearly show that the amount of the conjugate measured in the lungs at different times is much higher than for the unconjugated drug. Moreover, we also observed that the accumulation of the conjugate in the lung is also much higher than its concentration in the serum (plasma) at various times (Fig. 12). Results presented in Fig. 10, 11 and 12,

strongly indicate that the conjugation of an anticancer drug (e.g., Taxol) to the carrier of the present invention (e.g., Angiopep-1 or 2) modifies the biodistribution of the anticancer drug and its pharmacokinetics.

5 EXAMPLE 6

Inhibition of tumor growth *in vivo* (U-87)

The ability of conjugate to inhibit tumor growth was next evaluated in an *in vivo* model (Fig. 13). U-87 cells were therefore subcutaneously implanted in the right flank of mice and on day 3 post-implantation, mice were injected with the vehicle (DMSO/Ringer : 80/20; control), Taxol (5mg/kg) or Taxol-Angiopep-2 (10 mg/kg; equivalent to 5 mg of Taxol/kg (3 Taxol : 1 Angiopep-2)). We observed that the tumor growth inhibition was more pronounced in mice treated with the conjugate than in mice treated with the unconjugated anticancer drug.

In fact at day 17 post-implantation, tumor growth was inhibited by more than 75% by the conjugate whereas tumor growth was inhibited by only 34% using the unconjugated drug (Table 5). These results show that the conjugates described herein are more efficient than unconjugated Taxol at inhibiting tumor growth *in vivo*. Overall, a 2.2-fold tumor growth inhibition level was measured for the conjugate compared to the unconjugated drug.

Table 5. Inhibition of tumor growth with conjugates.

Groups	Tumor volume (mm ³) (mean ± sem)		Tumor growth	Inhibition (%)	T/C (%)
	Days post-injection		Δ (mm ³)		
	Day 0*	Day 14**			
Control	79 ± 7	289 ± 50	203 ± 47		100
Taxol (5mg/kg)	74 ± 5	219 ± 52	134 ± 55	34	66
TxlAn2 (3:1) (10mg/kg)	88 ± 9	144 ± 27	56 ± 32	73	27

Treatment equivalent to 5mg/kg of Taxol

* corresponds to 3 days post-implantation (first treatment)

** corresponds to 17 days post-implantation (after 4 treatments)

EXAMPLE 7**Mechanism of action of conjugates**

In Fig. 14, lung cancer cells (NCI-H460) were incubated for 24 hrs with either free Taxol (30 nM) or Tx1An2 conjugate (10 nM; equivalent to 30 nM of Taxol). After cells were labeled for β -tubulin by using a secondary antibody linked to FITC. Pictures were taken in visible and fluorescence modes. These results indicate that both Taxol and Taxol-Angiopep conjugate have similar effect on β -tubulin leading to its polymerization. Moreover, as indicated in Fig. 15, the addition of Taxol and Taxol-Angiopep conjugate induce a blockade of NCI-H460 cell in G2/M phase. Results obtained for the β -tubulin polymerization and cell cycle suggest that Tx1An conjugate has a similar mechanism of action on cancer cells than Taxol.

EXAMPLE 8**Effect on LRP-mediated RAP accumulation**

It was previously shown in international patent application No. PCT/CA2004/00011, that the receptor-associated protein (RAP) inhibited transcytosis of aprotinin in an *in vitro* model of the blood brain barrier. According to these data we proposed that the low-density lipoprotein related receptor (LRP) is involved in the penetration of aprotinin into the brain. Similar inhibition of Angiopep transport across an *in vitro* model of the blood-brain barrier was also obtained (data not show) suggesting that transcytosis of Angiopep across brain endothelial cell also involved LRP. LRP is a heterodimeric membrane receptor of 600 kDa composed of two subunits; the subunit- α (515 kDa) and the subunit- β (85 kDa). Immunodetection of LRP was then performed to assess whether this receptor is expressed in human primary brain tumors such as glioblastomas and in human brain metastasis from breast, lung and melanoma cancers (Fig. 16). Briefly, equal amount of protein homogenates from human primary brain tumors (glioblastomas) or human brain metastasis were separated by gel electrophoresis. After electrophoresis, proteins were transferred to PVDF membrane and LRP was immunodetected by using a monoclonal antibody directed against the subunit- α obtained from Cedarlane Laboratories (Hornby, ON, Canada). LRP was visualized by a secondary antibody directed against mouse IgG linked to horseradish peroxidase and chemiluminescence reagents.

Under the experimental conditions used, the subunit α of LRP was immunodetected at 515

kDa in glioblastoma U-87 cells. LRP was also detected in all human primary brain tumors and human brain metastases (Fig. 16). In contrast, megalin (LRP2) was detected in only one brain metastasis of the lung (not shown). The expression of LRP in the different patient-
5 Aprotinin conjugate in brain tumors. Overall, since LRP may be involved in the transport of the carrier described herein, these results indicate that the conjugates may also target cells and tumors which express this receptor.

In order to determine whether aprotinin and Angiopep transcytosis could involve LRP, their
10 impact on the uptake of the receptor-associated protein (RAP), an endogenous ligand for LRP, was determined (Fig. 17A). The uptake of RAP was measured in fibroblasts expressing LRP (MEF-1) and in fibroblasts that do not express LRP (PEA-13) (Fig. 17A and 17B). The addition of aprotinin inhibited the transport of [125 I]-RAP in positive LRP cells in a dose-dependent manner. In contrast, the RAP uptake in negative LRP cells was almost unaffected
15 by these aprotinin concentrations. In Fig. 17B the difference between the uptake of [125 I]-RAP measured in MEF-1 and PEA-13 was calculated and plotted as a function of aprotinin concentration. These results show that a portion of the LRP-dependent uptake of RAP could be reduced by aprotinin indicating that aprotinin could interact with this receptor. In a different experiment (Fig. 18), the uptake of [125 I]-RAP was also measured in the presence of
20 an excess of aprotinin and Angiopep. Results show that both aprotinin and Angiopep affect the LRP-dependent accumulation of [125 I]-RAP.

In summary, data obtained for the conjugates described herein indicate that the conjugation
25 of anticancer drugs to the carrier allows the anticancer drug to escape from P-gp action and therefore increase their potency (when conjugated with the carrier). These conjugates are active *in vitro* at inhibiting cancer cell proliferation. Moreover, results obtained on *in vivo* tumor growth indicate that the conjugation of anticancer drug to the carrier may increase their efficiency by bypassing P-gp, possibly targeting the receptor LRP or by modifying the pharmacokinetics or biodisponibility of the unconjugated drug.

30 Taken together, data described herein indicates that the conjugates may be used against primary tumors including breast, lung and skin cancers as wells as metastasis originating from primary tumors.

EXAMPLE 9**Improved formulation of Taxol-Angiopep conjugates**

Preliminary assays performed to assess the solubility of the different Tx1An conjugates indicated that all conjugates had a low solubility in aqueous solution (e.g., in Ringer/Hepes solution) due to the highly hydrophobic nature of Taxol. However, all of the conjugates were very soluble in dimethyl sulfoxide (DMSO)/Ringer (80%/20%). Different strategies were thus assessed to increase their solubility and to reduce the amount of DMSO necessary for their solubilization. Interestingly, we were able to completely remove DMSO from the formulation by using the solubilizer agent Solutol[®] HS15 (BASF). For example, Tx1An2 (3:1) at 5 mg/ml was efficiently solubilized in 20% Solutol[®] HS15 and Ringer/Hepes solution pH 5.5. As this agent has been approved for several drugs applications for intravenous (i.v.) and intraperitoneal (i.p.) administration, its use provides a commercial advantage to the formulations of the present invention.

Formulations of the present invention may thus comprise, for example, a) Taxol-Angiopep conjugates, b) Solutol[®] HS15 and c) an aqueous solution or buffer (e.g., Ringer/Hepes solution at a pH of 5 to 7). The concentration of Solutol[®] HS15 in the formulation may reach, for example, 30%. Concentration higher than 30% may also be useful. The concentration of conjugate may be determined based upon the dose required for efficiently treating a patient.

EXAMPLE 10**Blood kinetics of the improved formulations**

For tissue distribution and blood kinetic studies, iodinated [¹²⁵I]-conjugates (i.e., [¹²⁵I]-Tx1An conjugates) and [³H]-Taxol were used. Briefly, Tx1An2 conjugates (1 mg) were radioiodinated using iodobeads and Tx1-[¹²⁵I]-An2. Conjugate was then purified using a column containing resource RPC resin. Free iodine was removed by washing the column thoroughly with 20% acetonitrile. During column washes radioactivity was counted to assess the decrease in free iodine. The Tx1-[¹²⁵I]-An2 conjugate was then pulled-down by a 100% acetonitrile wash. Acetonitrile was then evaporated and the iodinated conjugate was diluted in 100% DMSO (100 µL). An aliquot of the radioiodinated conjugate was then injected in

HPLC and fractions were collected to verify that the radioactivity was associated to the fractions corresponding to the conjugates.

Blood kinetics were assessed after intravenous ((i.v.) tail vein), intraperitoneal (i.p.) and subcutaneous (s.c.) injections performed on awake mice (Fig. 19). Briefly Tx1An2 (3:1) conjugate was diluted in DMSO/Ringer-Hepes (80/20) or in Solutol®/Ringer-Hepes (20/80), Tx1-[¹²⁵I]-An2. Injections of CD-1 mice with the formulations were then performed to obtain a 10 mg/kg concentration. After injections blood fractions (50µL) were collected at the tail end and radioactivity was directly assessed. Using the same protocol, Taxol blood kinetic was also determined using [³H]-Taxol. Taxol was dissolved in DMSO/Ringer-Hepes (80/20) at a concentration allowing a 5 mg/kg injection and [³H]-Taxol was then added (2.5µCi/injection). After injections blood fractions were collected at the tail end, scintillation cocktail was added and radioactivity was counted in a Packard counter. Results of this experiment are illustrated in Fig. 19A, Fig. 19B and in Fig. 19C and are summarized in Table 6 below.

In summary, results of Fig. 19 and Table 6 show that Tx1An2 bioavailability is much higher than Taxol bioavailability. For example, the $AUC_{(0-24hrs)} \text{Tx1An2} / AUC_{(0-24hrs)} \text{Taxol}$ is 169 (i.e., 203.3/1.2).

In terms of Taxol, the $AUC_{(0-24hrs)} \text{Tx1An2} / AUC_{(0-24hrs)} \text{Taxol}$ is 84.7 (i.e., 101.65/1.2). Since there is three Taxol molecules on each molecule of Angiopep-2, the amount of Taxol represents about 0.5 of Angiopep's molecular weight (i.e., $3 \times 854 / 5301$). Therefore the AUC of the conjugate (i.e., 203) has to be multiplied by 0.5 in order to be expressed in term of Taxol.

In addition the blood biodisponibility of Tx1An2 conjugates is equivalent after intravenous and intraperitoneal injections whereas this is not the case for Taxol. Finally, results of Fig. 19 and Table 6 indicate that the blood biodisponibility of Tx1An2 is higher when Solutol® is used as solubilizer compared to DMSO.

AUC (0-24h) ($\mu\text{g}\cdot\text{h}$ per mL)	Intravenous injection	Intraperitoneal injection	Subcutaneous injection
TxlAn2 (3:1) (DMSO) (10mg/kg)	98.1	34.7	15.5
TxlAn2 (3:1) (Solutol [®]) (10mg/kg)	203.3	211.3	74.5
Taxol (DMSO) (5mg/kg)	1.2	0.09	0.14

Table 6: Area under curves from 0 to 24 hours were calculated using GraphPad software.

EXAMPLE 11

Tissue distribution

- 5 TxlAn2 tissue distribution was evaluated in normal CD-1 mice after tail vein intravenous injection of 10 mg/kg TxlAn2 solubilized in Solutol[®]/Ringer-Hepes (20%/80%) or in DMSO/Ringer-Hepes (80/20). Briefly CD-1 mice were injected via the tail vein with a formulation of TxlAn2 solubilized in Solutol[®] or DMSO and also containing Txl-[¹²⁵I]-An2. At predetermined time points a blood sample was collected and anesthetized mice were
- 10 perfused with cold PBS. Tissues were then excised and radioactivity was counted in a gamma counter.

Results of Fig. 20 show that the use of Solutol[®] allows a higher distribution of TxlAn2 (3:1) conjugate in most tissues.

15

EXAMPLE 12

Distribution in brain tumors

- In an attempt to evaluate the distribution of TxlAn2 (3:1) conjugate in a brain tumor model, nude mice were intracerebrally implanted with NCI-H460 lung cancer cells. Ten days after
- 20 implantation, mice weight loss was significant indicating that the brain tumors were well established. Taxol, TxlAn2 (3:1) and TxlAn2 (2:1) tissue distributions were evaluated (Fig. 21), as precedently described.

- Mice were thus given an intravenous injection of either Taxol (5mg/kg) solubilized in
- 25 DMSO or TxlAn2 (3:1) (10mg/kg) or TxlAn2 (2:1) (12.5mg/kg) each solubilized in

Solutol®. After 10 minutes, mice were perfused on ice using cold PBS, organs were collected and radioactivity was measured. To evaluate the difference of accumulation between normal brain and brain tumor, brains were cut in half with the right hemisphere (site of injection of the tumor cells) corresponding to the tumoral brain and the left hemisphere to the normal brain.

Results of Fig. 21A and Fig. 21B show that Tx1An2 (3:1) conjugates present a higher distribution in brain tumor compared to normal brain (2-fold increase) whereas no difference is observed for Taxol distribution between normal and tumoral brain. Tx1An2 (3:1) conjugate distribution is much higher than Taxol distribution in brain tumor (10-fold increase) and was also higher than Tx1An2 (2:1) distribution (4.5-fold).

EXAMPLE 13

Effect of Taxol-Angiopep conjugates improved formulation on s.c. tumor growth.

In vivo studies were conducted to determine whether the improved formulation comprising the Taxol-Angiopep conjugate could inhibit lung cancer cell (NCI-H460) growth or glioblastoma cells (U87) growth in an *in vivo* model of mice implanted subcutaneously with these cancer cells.

Briefly, mice received a subcutaneous injection of 2.5×10^6 human U87 glioma cells or NCI-H460 cells. When tumor growth was observed, mice received treatment with free Taxol, Taxol-Angiopep conjugates or vehicle by i.v. or i.p. injections. Treatments were then administered twice a week until animals were sacrificed. Mice were monitored every day for clinical symptoms and weight loss. Tumor volume was estimated with a kaliper and the following equation (tumor volume = $\pi/2 \times (\text{length (mm)} \times \text{width}^2 \text{ (mm)})$).

In the first subcutaneous tumor growth study, NCI-H460 cells were implanted in mice right flank (Fig. 22A). Mice received the vehicle, Taxol or Taxol-Angiopep-2 (3:1) conjugate formulation by i.v. injections in the tail vein or i.p. injections. Conjugates were administered at an equivalent of 10 mg/kg of Taxol. Results presented in Fig. 22 show that the improved formulation of Tx1An2 (3:1) conjugate containing 20% Solutol® HS15 in Ringer/Hepes solution (pH 5.5) caused a much stronger inhibition of NCI-H460 tumor growth than Taxol.

These results are also summarized in Table 7 below

Molecules	Tumor volume (mm ³)		Tumor growth	T/C (%)
	Days post-injection		Δ (mm ³)	
	Day 0	Day 11		
Solutol	118 \pm 11	714 \pm 71	596 \pm 62	100
Taxol i.v.	112 \pm 12	483 \pm 63	371 \pm 58	62
TxlAn-2 (3:1) i.v.	112 \pm 18	236 \pm 32	124 \pm 14	21
TxlAn-2 (3:1) i.p.	105 \pm 14	221 \pm 50	116 \pm 37	19
TxlAn-2 (2:1) i.p.	111 \pm 18	215 \pm 70	104 \pm 53	17

Table 7

- 5 These results indicate that the TxlAn conjugates are more potent than Taxol at inhibiting tumor growth in an *in vivo* setting. In addition, similar results were obtained whether the conjugate was administered i.v. or i.p. Finally, similar results were also obtained TxlAn conjugates comprising 2 or 3 Taxol molecules.

10

EXAMPLE 14

Effect of Taxol-Angiopep conjugates improved formulation on s.c. tumor growth.

- 15 In a further study, the effect of TxlAn2 (3:1) conjugate formulations on s.c. NCI-H460 or U87 growth was evaluated. Mice were treated by i.p. injections with the improved formulation at 20 mg/kg/day for five consecutive days or by infusion with the implantation of Alzet mini-osmotic pump at a dose of 2 mg/kg/day for 14 days. As shown in Fig. 23A and Fig. 23B, the response of mice to TxlAn2 (3:1) conjugate formulation was higher when mice received the improved formulation by infusion.

- 20 These *in vivo* experiments clearly show the efficacy of the improved formulation against tumor growth of glioblastoma or lung cancer cells. Similar experiments also indicate the efficacy of these improved formulations in prolonging survival of animals (data not shown).

- 25 The content of each publication, patent and patent application mentioned in the present application is incorporated herein by reference.

Although the present invention has been described in details herein and illustrated in the accompanying drawings, it is to be understood that the invention is not limited to the embodiments described herein and that various changes and modifications may be effected without departing from the scope or spirit of the present invention.

CLAIMS:

1. A pharmaceutical composition for reducing the growth of a cell or for the detection of a cell, the pharmaceutical composition comprising:

- 5 a. a conjugate comprising a carrier selected from the group consisting of aprotinin, a biologically active aprotinin fragment, Angiopep-1, Angiopep-2 and biologically active analogs, derivatives or fragments thereof and a label or a small molecule drug able to reduce the growth of a cell and;
- b. a pharmaceutically acceptable carrier.

10 2. The pharmaceutical composition of claim 1, further comprising a solubilizer.

3. The pharmaceutical composition of claim 2, wherein said solubilizer is a poly-oxyethylene ester of fatty acid.

15 4. The pharmaceutical composition of claim 3, wherein said poly-oxyethylene ester of fatty acid is Solutol® HS-15.

20 5. The pharmaceutical composition of any of claims 1 to 4, wherein said cell is a tumor cell.

25 6. A pharmaceutical composition comprising:

- a. a conjugate comprising a carrier selected from the group consisting of aprotinin, a biologically active aprotinin fragment, Angiopep-1, Angiopep-2 and biologically active analogs, derivatives or fragments thereof and a small molecule drug or label,
- b. a pharmaceutically acceptable carrier, and;
- c. a solubilizer.

30 7. The pharmaceutical composition of claim 6, wherein said solubilizer is a poly-oxyethylene ester of fatty acid.

8. The pharmaceutical composition of claim 7, wherein said poly-oxyethylene ester of fatty acid is Solutol® HS-15.

9. The use of a carrier selected from the group consisting of aprotinin, a biologically active aprotinin fragment, Angiopep-1, Angiopep-2 and biologically active analogs, derivatives or fragments thereof or the pharmaceutical composition of any one of claims 1 to 8 for promoting accumulation of a molecule selected from the group consisting of a label, a protein, a peptide and a small molecule drug inside a cell.
10. The use as defined in claim 9, wherein said cell is a cell expressing P-gp (at a cell surface).
11. The use as defined in claim 9 or 10, wherein said cell is a brain cell, a lung cell, a breast cell, a kidney cell, an eye cell and a liver cell.
12. The use as defined in claim 11, wherein said cell is a tumor cell.
13. The use as defined in any one of claims 10 to 12 wherein said cell is located outside of a brain of an individual.
14. The use as defined in any one of claims 10 to 12, wherein said cell is located inside a brain of an individual.
15. The use as defined in claim 13 or 14, wherein said tumor cell is a brain tumor cell.
16. The use as defined in claim 15, wherein said brain tumor cell originates from a glioblastoma.
17. The use as defined in claim 13 or 14, wherein said tumor cell is a lung tumor cell.
18. The use of a carrier selected from the group consisting of aprotinin, a biologically active aprotinin fragment, Angiopep-1, Angiopep-2 and biologically active analogs, derivatives or fragments thereof or the pharmaceutical composition of any one of claims 1 to 8 for reducing the growth of a cell.

19. The use as defined in claim 18, wherein said carrier is conjugated with a drug able to reduce the growth of a cell.

20. The use as defined in claim 19, wherein said cell is a tumor cell or an endothelial cell.

5

21. The use as defined in claim 20, wherein said tumor cell is a cell expressing P-gp.

22. The use as defined in claim 20 or 21, wherein said tumor cell is a brain tumor cell, a lung tumor cell, a breast tumor cell, a kidney tumor cell or an eye tumor cell.

10

23. The use as defined in claim 22, wherein said cell is located outside of a brain of an individual.

24. The use as defined in claim 22, wherein said cell is located inside a brain of an individual.

15

25. The use as defined in claim 23 or 24, wherein said brain tumor cell originates from (or is) a glioblastoma.

20 26. The use as defined in any one of claims 19 to 25, wherein said drug is an anticancer drug.

27. The use as defined in claim 26, wherein said anticancer drug is selected from the group consisting of Taxol, vinblastine, vincristine, etoposide, doxorubicin, cyclophosphamide, Taxotere, melphalan, chlorambucil and combination thereof.

25

28. The use of a carrier selected from the group consisting of aprotinin, a biologically active aprotinin fragment, Angiopep-1, Angiopep-2 and biologically active analogs, derivatives or fragments thereof or the pharmaceutical composition of any one of claims 1 to 8 or a conjugate comprising the carrier and a compound selected from the group consisting of a label, a protein, a peptide and a small molecule drug for modifying the pharmacokinetics of the compound.

30

29. The use as defined in claim 28, wherein said small molecule drug is an anticancer drug.

30. The use as defined in claim 29, wherein said anticancer drug is selected from the group consisting of Taxol, vinblastine, vincristine, etoposide, doxorubicin, cyclophosphamide, Taxotere, melphalan, chlorambucil and combination thereof.

5 31. The use as defined in claim 29 or 30, wherein said anticancer drug is conjugated with said carrier thereby forming a conjugate.

32. The use as defined in claim 31, wherein said conjugate comprises at least one anticancer drug molecule for each carrier molecule.

10

33. The use as defined in claim 31, wherein said conjugate comprises at least two anticancer drug molecules for each carrier molecule.

15

34. The use as defined in claim 31, wherein said conjugate comprises at least three anticancer drug molecules for each carrier molecule.

20

35. The use as defined in any one of claims 28 to 34, wherein said carrier promotes accumulation of said compound in a tissue selected from the group consisting of kidney, liver, eye and lungs of an individual.

36. The use as defined in any one of claims 28 to 35, wherein said carrier promotes accumulation of said compound in a brain (brain tissue) of an individual.

25

37. The use as defined in claim 36, wherein said brain is a tumoral brain.

38. The use as defined in claim 36, wherein said brain comprises a lung cancer cell.

30

39. The use as defined in any one of claims 29 to 34, wherein said carrier promotes accumulation of said drug in a cancer cell.

40. The use as defined in any one of claims 28 to 34, wherein said carrier modifies the bioavailability of the compound.

41. The use as defined in any one of claims 28 to 34, wherein said carrier modifies the tissue distribution of the compound.
42. The use as defined in any one of claims 29 to 34, wherein said carrier increases an anti-tumor growth effect of the anticancer drug.
43. The use as defined in claim 28, wherein said small molecule drug is a drug able to reduce the growth of a cell.
44. The use as defined in claim 43, wherein said cell is tumor cell.
45. The use as defined in claim 44, wherein said tumor cell expresses P-gp.
46. The use as defined in claim 44, wherein said tumor cell is a multiple drug resistant cell.
47. The use as defined in claim 43, wherein said cell is an endothelial cell.
48. The use as defined in any one of claims 44 to 46, wherein said tumor cell is a brain tumor cell, a lung tumor cell, a breast tumor cell, a kidney tumor cell or an eye tumor cell.
49. The use as defined in any one of claims 44 to 46, wherein said cell is located outside of a brain of an individual.
50. The use as defined in any one of claims 44 to 46, wherein said cell is located inside a brain of an individual.
51. The use as defined in claim 49, wherein the brain tumor cell is an extracranial metastasis from a glioblastoma.
52. The use of a conjugate comprising a) a carrier selected from the group consisting of aprotinin, a biologically active aprotinin fragment, Angiopep-1, Angiopep-2 and biologically active analogs, derivatives or fragments thereof and b) at least one small molecule drug or label in the making of a pharmaceutical composition or medicament for

modifying the pharmacokinetics of said small molecule drug or label or for increasing an anti-tumor growth effect of said small molecule drug.

53. The use of a carrier selected from the group consisting of aprotinin, a biologically active aprotinin fragment, Angiopep-1, Angiopep-2 and biologically active analogs, derivatives or fragments thereof in the making of a pharmaceutical composition or medicament for modifying the pharmacokinetics of said small molecule drug or label or for increasing an anti-tumor growth effect of said small molecule drug.
54. A method for modifying the pharmacokinetics of a compound selected from the group consisting of a label, a protein, a peptide and a small molecule drug, the method comprising conjugating the compound with a carrier selected from the group consisting of aprotinin, a biologically active aprotinin fragment, Angiopep-1, Angiopep-2 and biologically active analogs, derivatives or fragments thereof, thereby forming a conjugate and administering said conjugate to an individual in need.
55. The method of claim 54, wherein one molecule of said compound is conjugated for each carrier molecule.
56. The method of claim 54, wherein at least two molecules of said compound is conjugated for each carrier molecule.
57. The method of claim 54, wherein at least three molecules of said compound is conjugated for each carrier molecule.
58. The method of any one of claims 54 to 57, wherein said compound is a small molecule drug.
59. The method of claim 58, wherein said small molecule drug is an anticancer agent.
60. The method of any one of claims 54 to 57, wherein said compound is a label.

61. The method of any one of claims 54 to 60, wherein said individual in need is an individual having a tumor.
- 5 62. The method of claim 61, wherein said tumor is a brain tumor.
63. The method of claim 61, wherein said tumor is an extracranial brain tumor.
64. The method of claim 61, wherein said tumor comprises a tumor cell expressing P-gp.
- 10 65. The method of claim 62, wherein said brain tumor is a primary brain tumor.
66. The method of claim 62, wherein said brain tumor is of a different tissue origin than brain tissue.
- 15 67. The method of claim 66, wherein said brain tumor originates from a lung tumor, a breast tumor, a colorectal tumor, a tumor of an urinary organ or from a melanoma.
68. The method of any one of claims 54 to 66, wherein the method promotes the accumulation of the compound within a cell.
- 20 69. The method as defined in any one of claims 54 to 66, wherein said carrier modifies the bioavailability of the compound.
70. The method as defined in any one of claims 54 to 66, wherein said carrier modifies the tissue distribution of the compound.
- 25 71. The method of any one of claims 59 to 66, wherein the method increases an anti-tumor growth effect of the anticancer drug.
- 30 72. The method as defined in claim 58, wherein said small molecule drug is a drug able to reduce the growth of a cell.
73. The method as defined in claim 72, wherein said cell is a tumor cell.

74. The method as defined in claim 72, wherein said cell is an endothelial cell.

75. The method of claim 73, wherein said tumor cell is located inside the brain of an individual.

76. The method of claim 75, wherein said tumor cell originates from a lung cancer, from a breast cancer, a colorectal tumor, a tumor of an urinary organ or from a melanoma.

77. The method of claim 73, wherein said tumor cell is located outside the brain of an individual.

78. A method of treating or diagnosing a cancer or a metastatic cancer, the method comprising administering a conjugate comprising a) a carrier selected from the group consisting of aprotinin, a biologically active aprotinin fragment, Angiopep-1, Angiopep-2 and biologically active analogs, derivatives or fragments thereof and b) an anticancer drug or a label to an individual having an extracranial tumor, a primary brain tumor or a brain tumor of metastatic origin.

79. The method of claim 78, wherein said individual has an extracranial tumor.

80. The method of claim 79, wherein said extracranial tumor is a lung tumor.

81. The method of claim 78, wherein said extracranial tumor is an extracranial metastasis from a brain tumor.

82. The method of claim 79, wherein said extracranial tumor is a metastasis from a brain tumor.

83. The method of claim 82, wherein said metastasis from a brain tumor is a glioblastoma.

84. The method of claim 78, wherein said individual has a brain tumor of metastatic origin.

85. The method of claim 84, wherein said brain tumor of metastatic origin is from a lung tumor.

86. The method of claim 84, wherein said brain tumor of metastatic origin is from a breast tumor.

5

87. The method of claim 84, wherein said brain tumor of metastatic origin is from a melanoma.

10

88. The method of claim 84, wherein said brain tumor of metastatic origin is from a colorectal tumor.

89. The method of claim 84, wherein said brain tumor of metastatic origin is from a tumor of an urinary organ.

15

90. The method of any one of claims 78 to 89, wherein said tumor comprises a tumor cell expressing P-gp.

91. The method of any one of claims 78 to 89, wherein said tumor comprises a tumor cell expressing LRP.

20

92. The method of claim 90, wherein said tumor cell further expresses LRP.

1/28

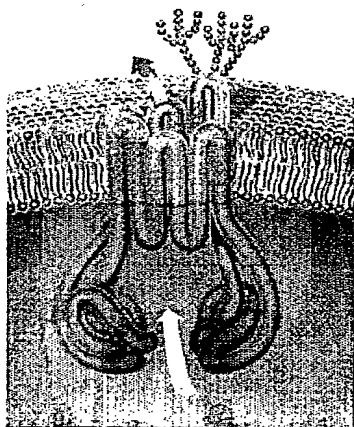
Angiopep-1 vs Angiopep-2

Angiopep-1:

TFFYGGCRGKRNNFKTEEY

Angiopep-2:

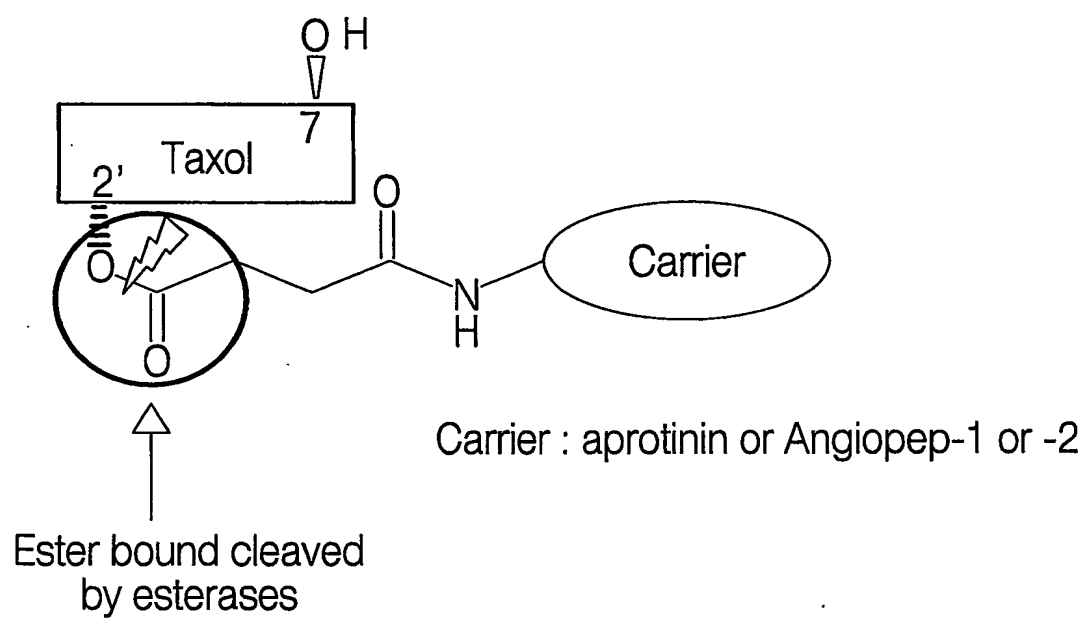
TFFYGGSRGKRNNFKTEEY

Fig-1

- Drug efflux pump
- Highly expressed at the blood-brain barrier
- Limits the passage of many drug toward the brain

Fig-2

2/28

Fig-3

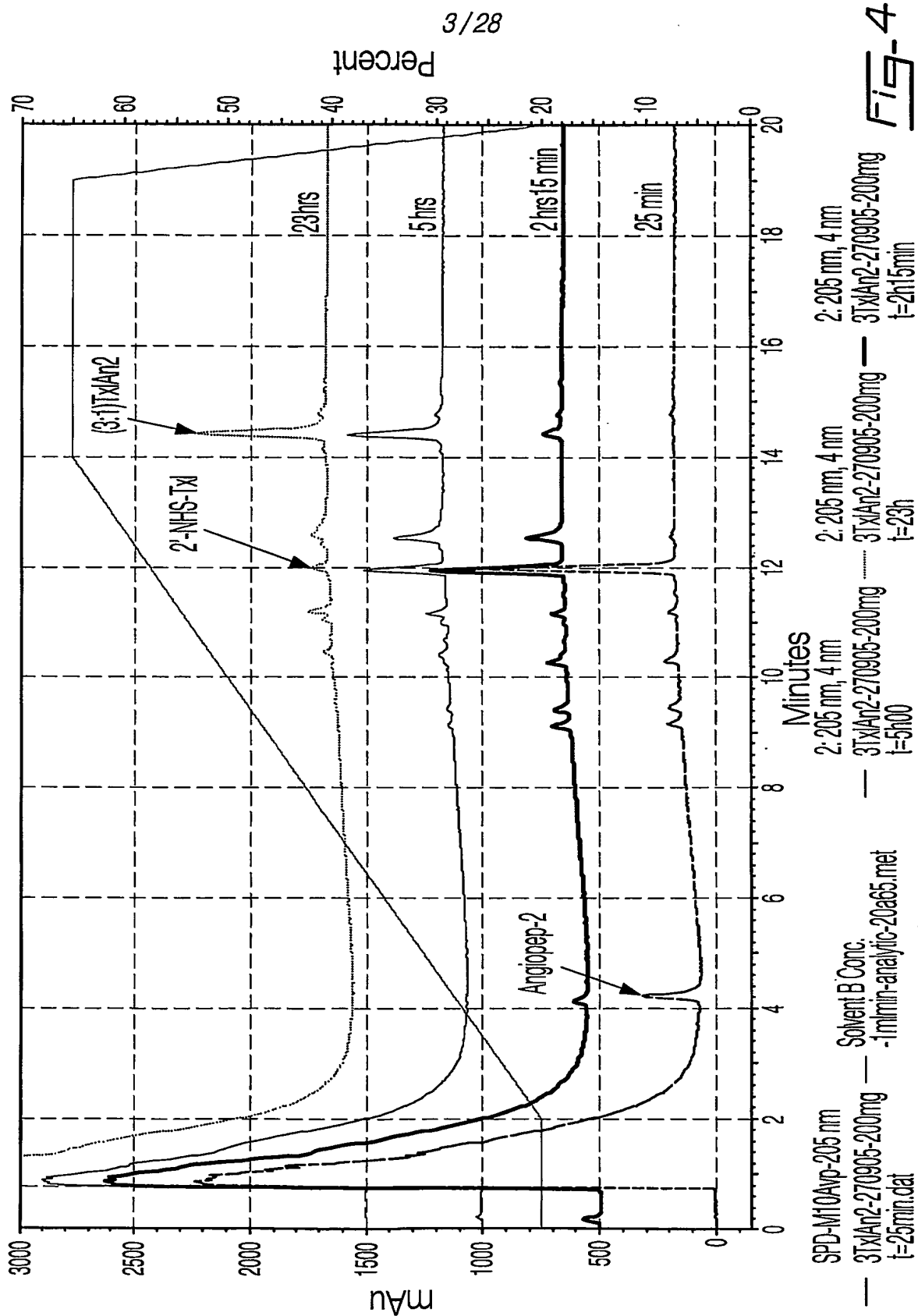


Fig-4

4/28

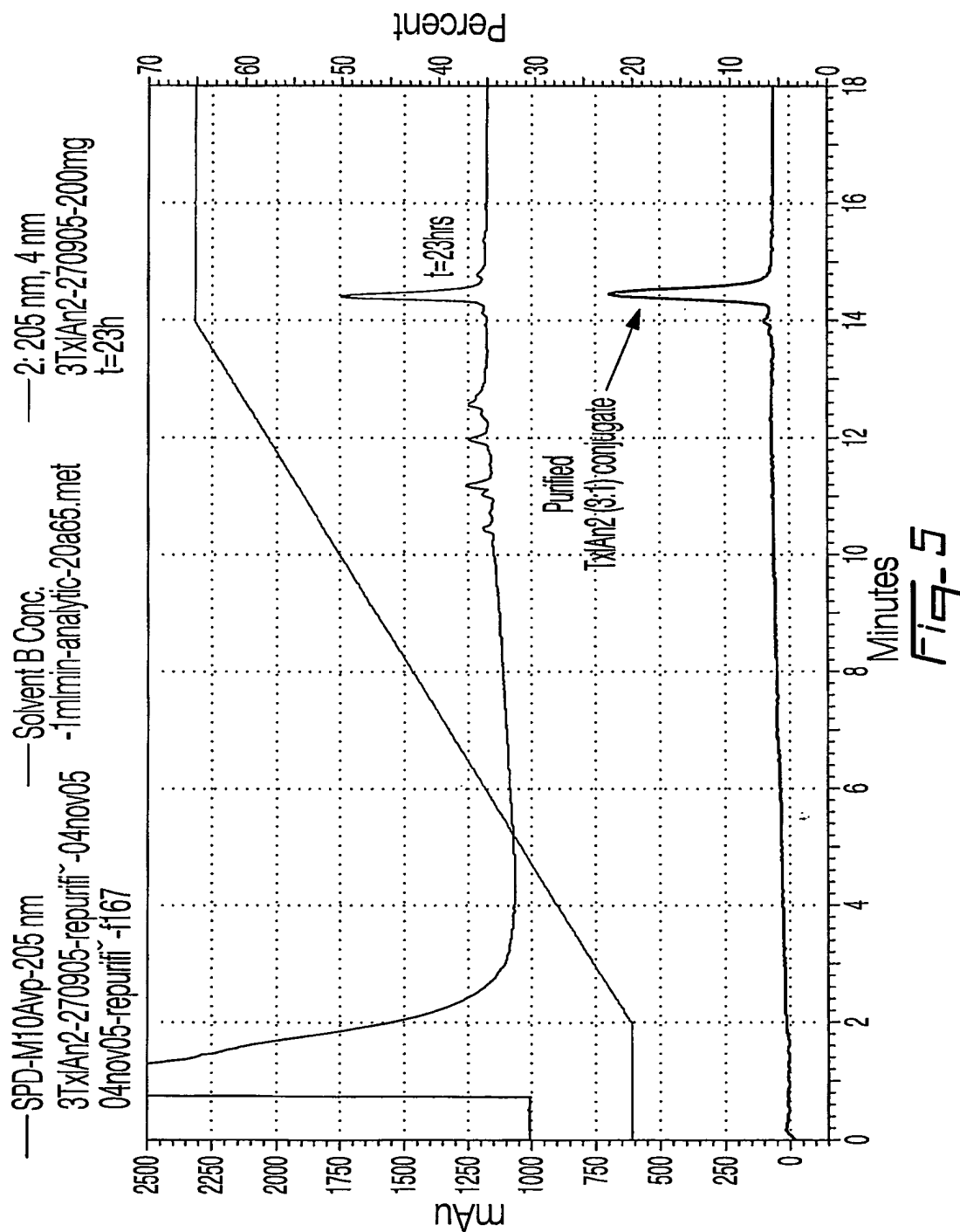
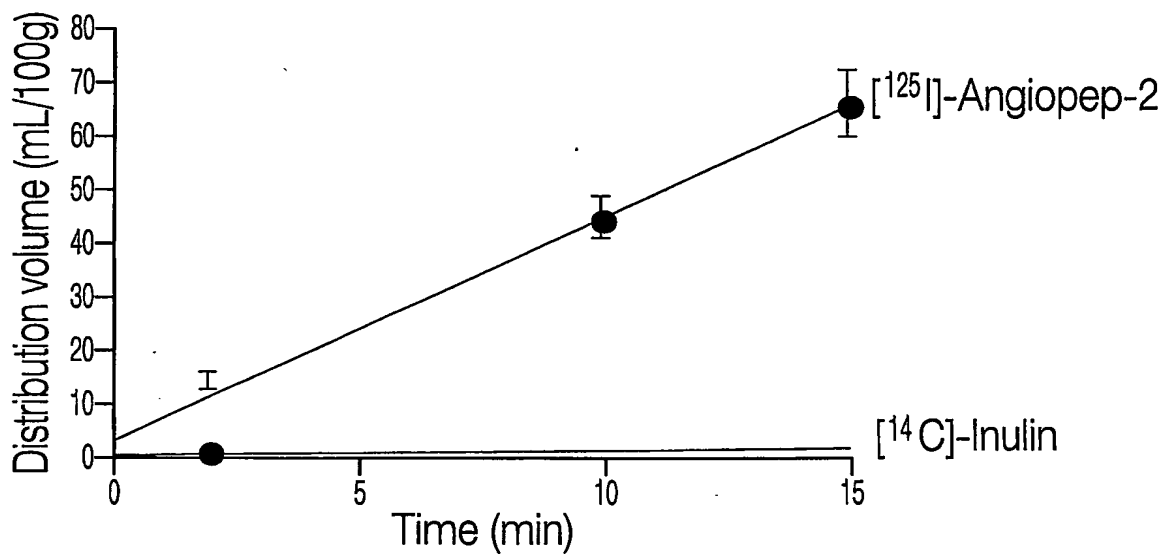
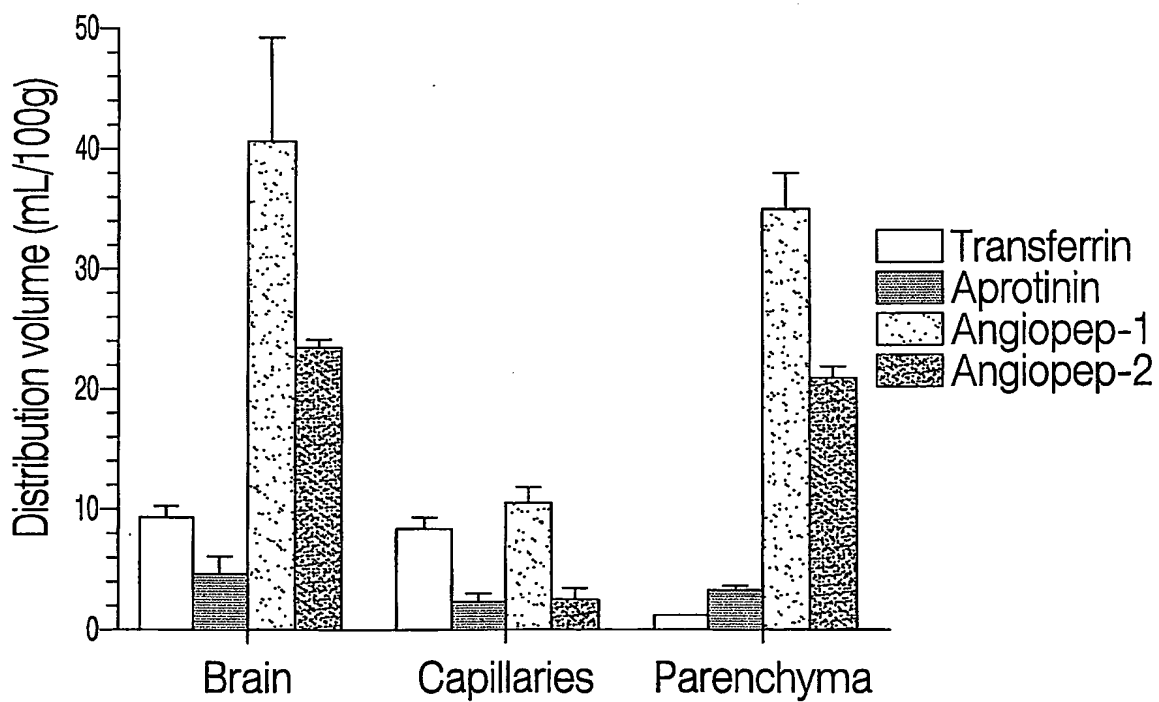


Fig-5

5/28

Fig-6Fig-7

6/28

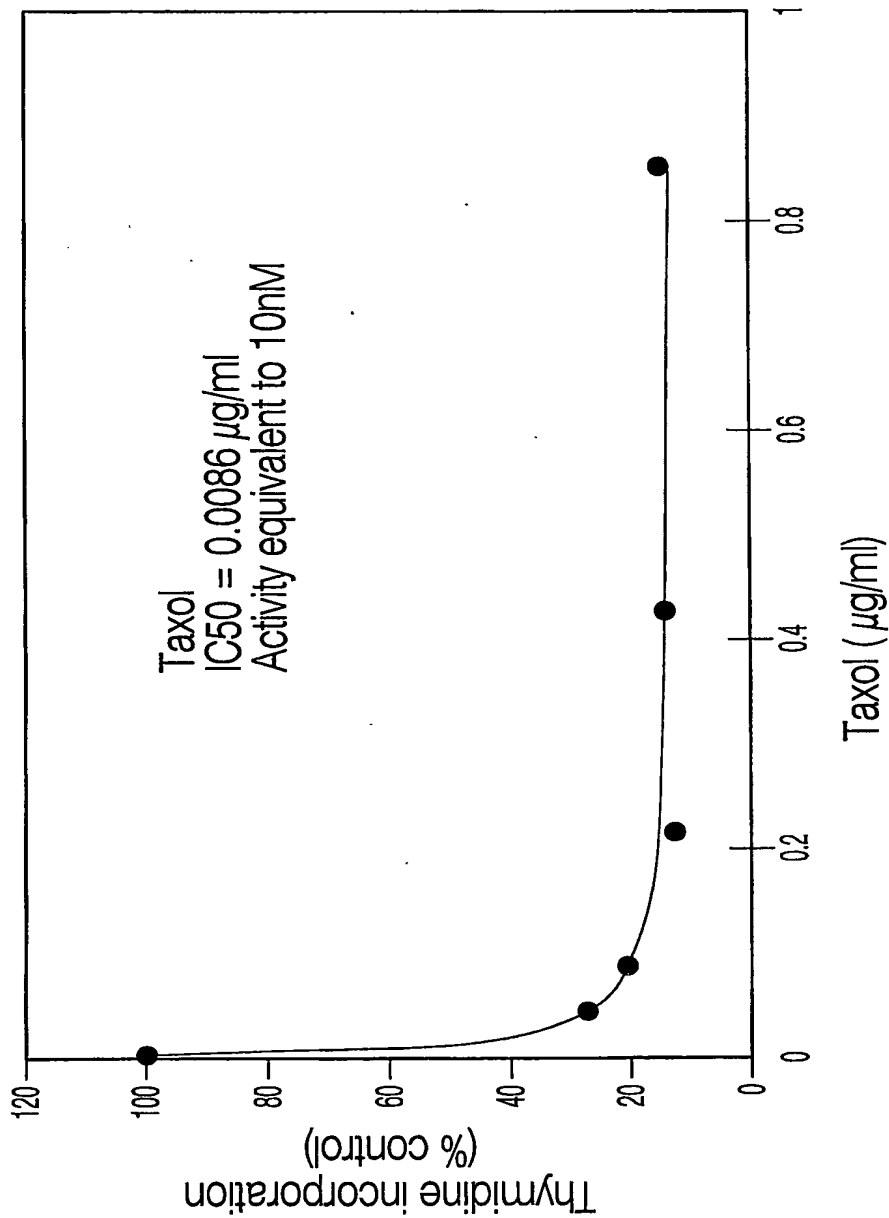
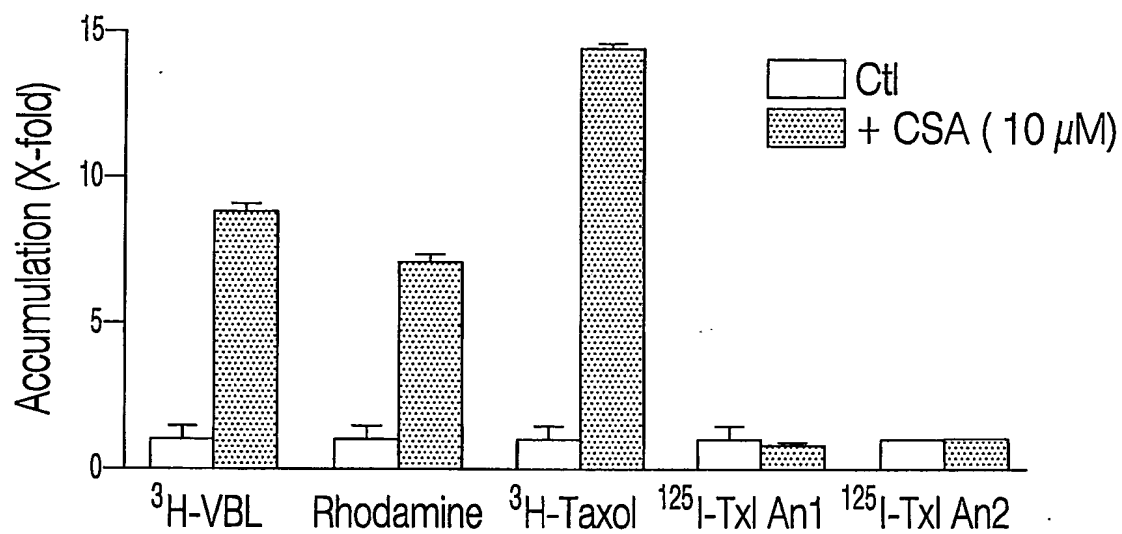
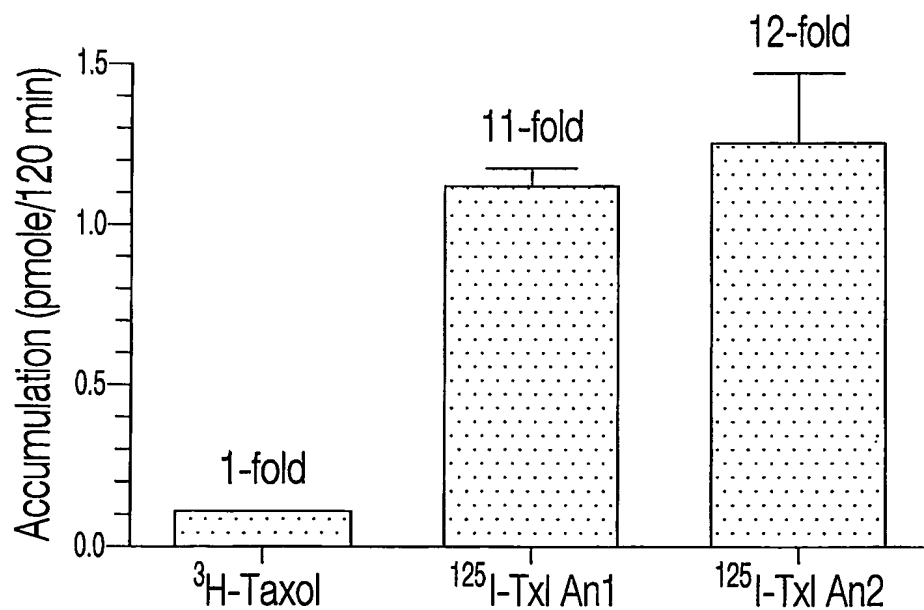


Fig. 8

7/28

Fig- 9AFig- 9B

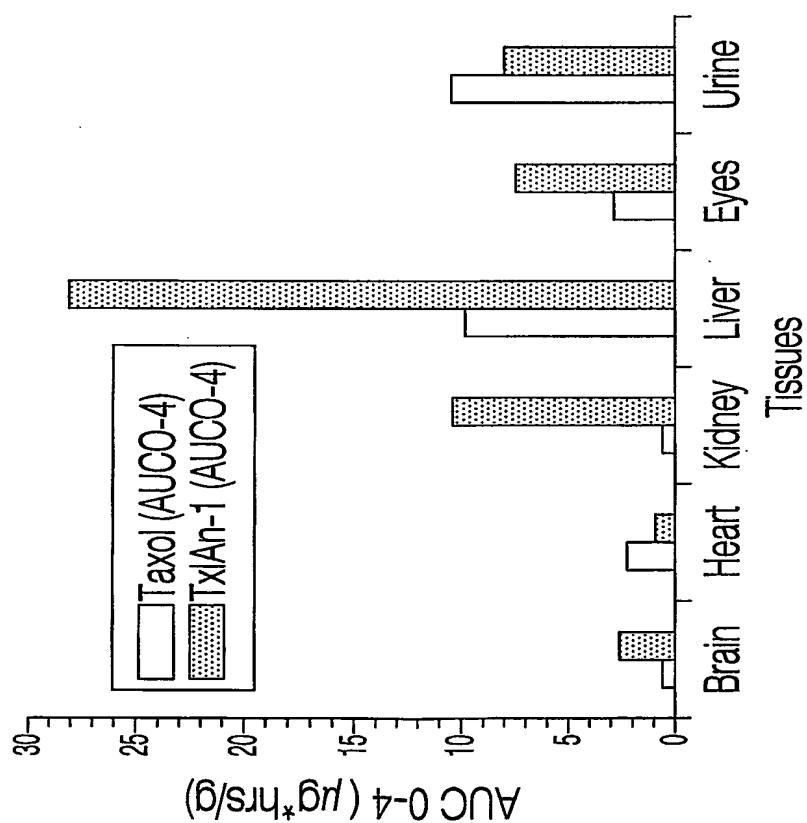


Fig-10A

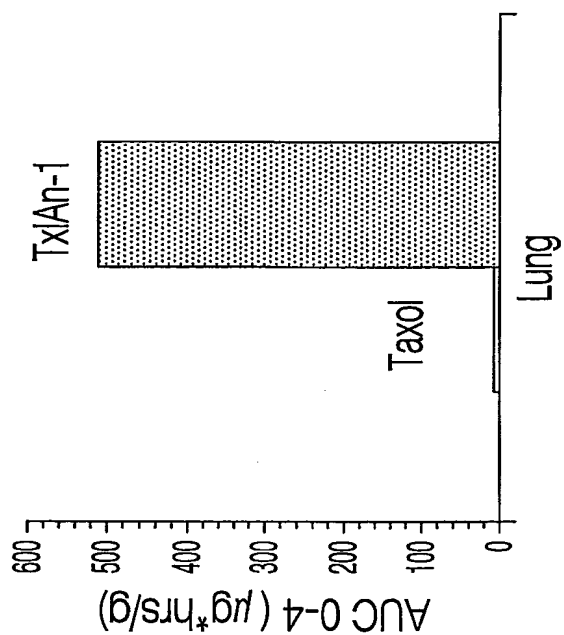
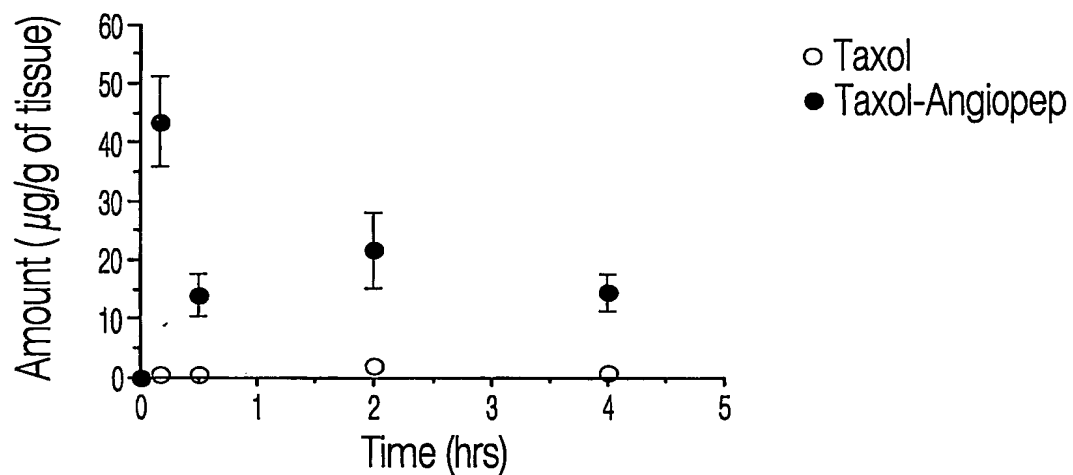
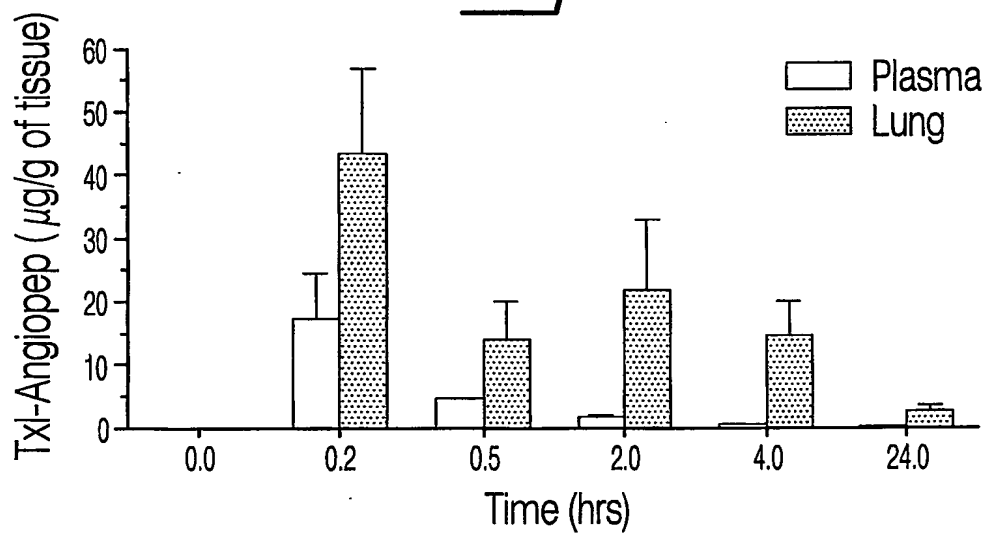
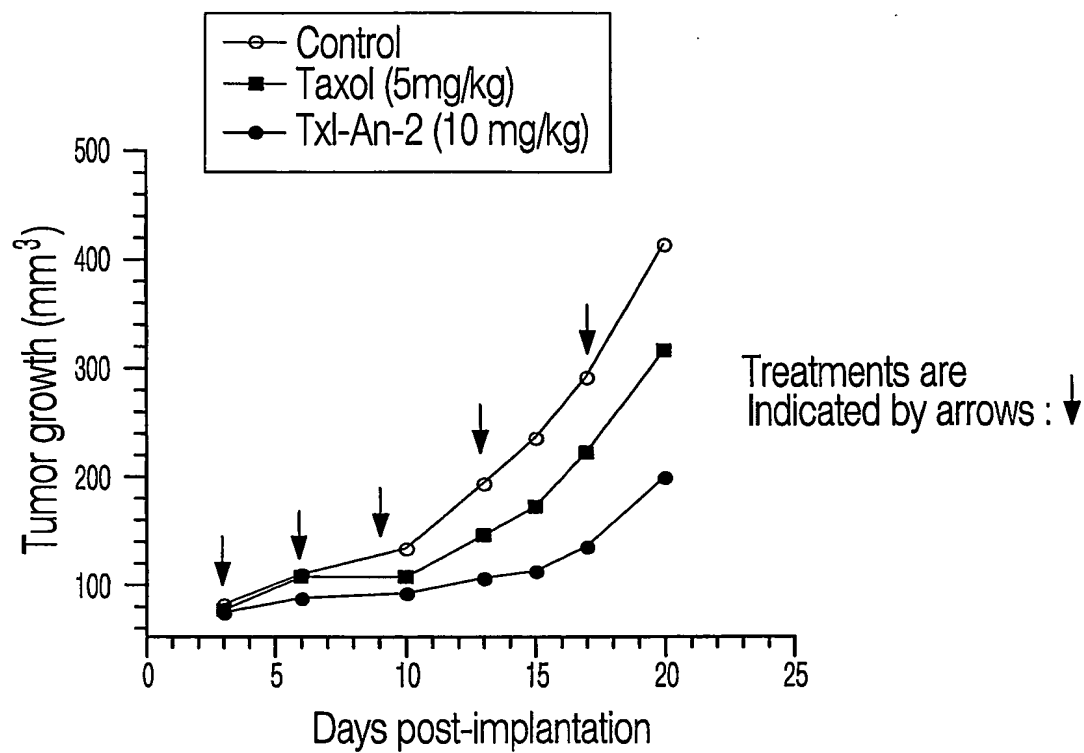


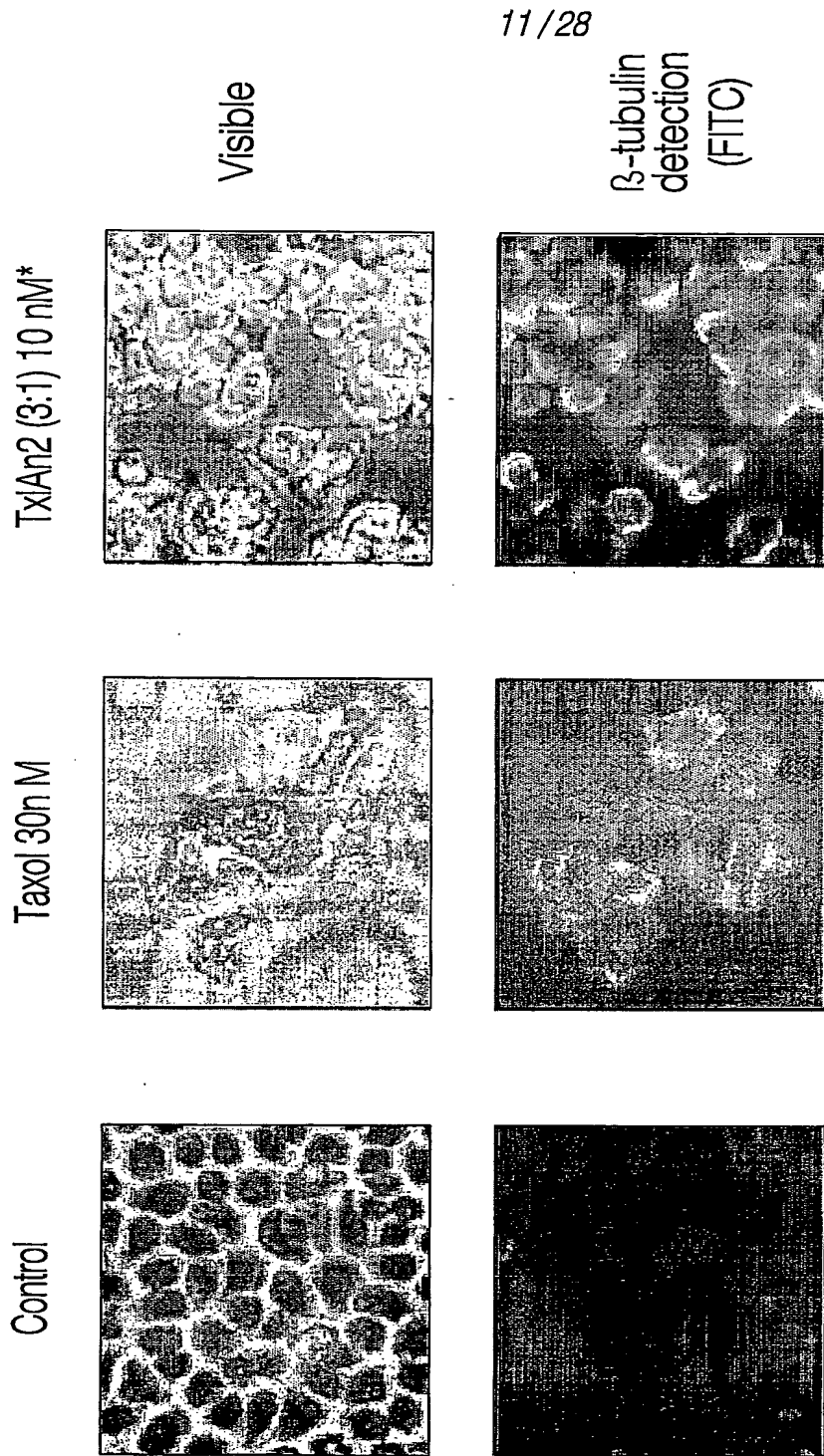
Fig-10B

9/28

Fig-11Fig-12

10/28

Fig-13



* Tx/An2 (3:1) conjugate equivalent to 30 nM of Taxol (24 hrs treatment)

Fig-14

12/28

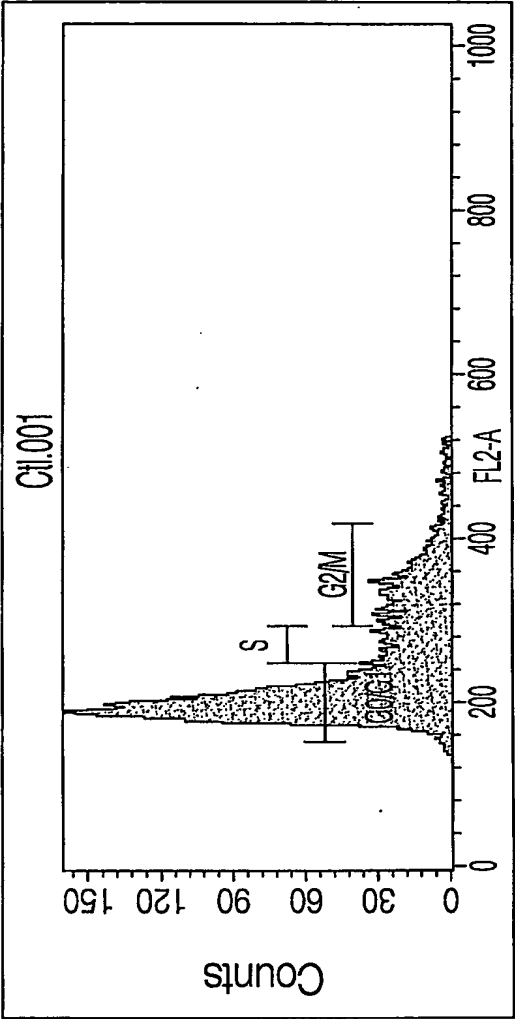


Fig-15A

File: Ctl001			
Sample Id: Ctl			
Patient ID:			
Acquisition Date: 03-Apr-06			
Gate: G2			
Marker	Events	% Gated	
All	9035	100.00	
G0-G1	6273	69.43	
S	985	10.90	
G2M	1747	19.34	

Control

13/28

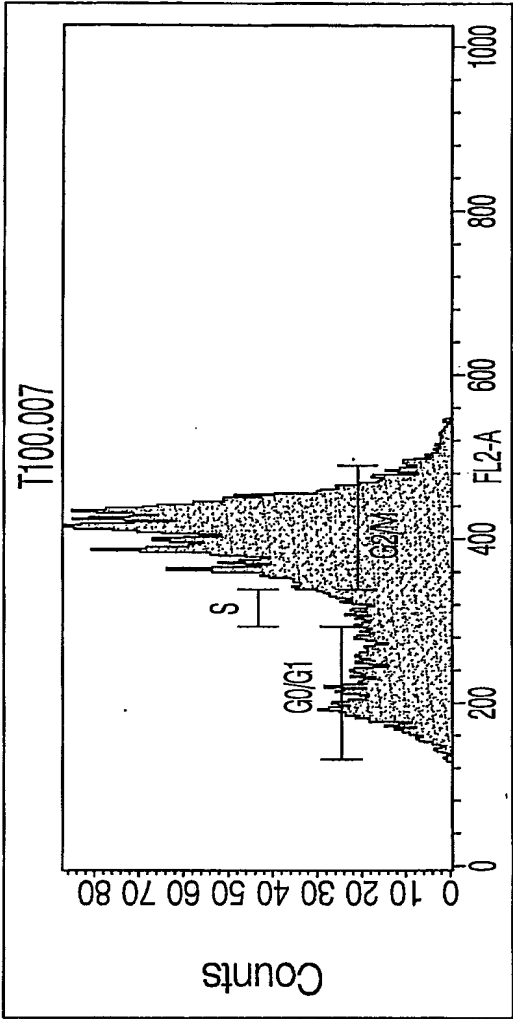


Fig-15B

File: T100.007		
Sample ID: T100		
Patient ID:		
Acquisition Date: 03-Apr-06		
Gate: G2		
Marker	Events	% Gated
All	9356	100.00
G0-G1	2104	22.49
S	821	8.78
G2/M	6373	68.12

Taxol
(100nM)

14/28

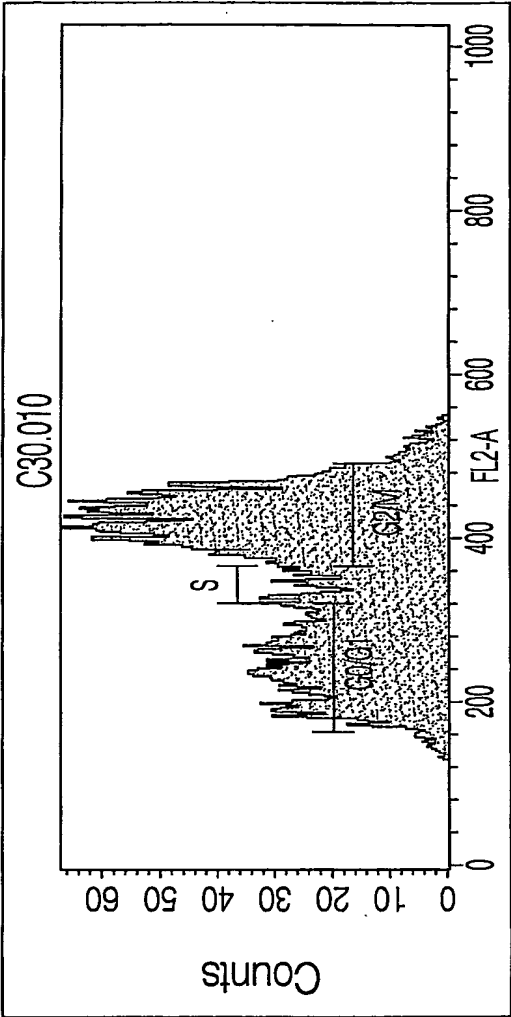


FIG-15C

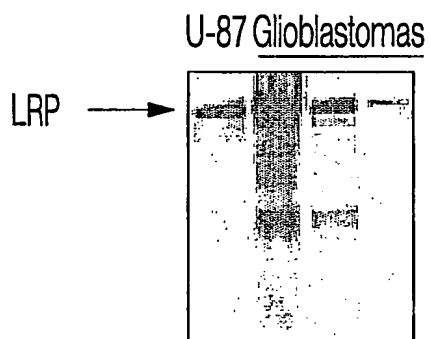
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Sample ID: C30			
Patient ID:			
Acquisition Date: 03-Apr-06			
Gate: G2			
Marker	Events	% Gated	
All	9255	100.00	
G0-G1	3379	36.51	
S	880	9.51	
G2M	4851	52.41	

Tx/An2(3:1)
(30nM)

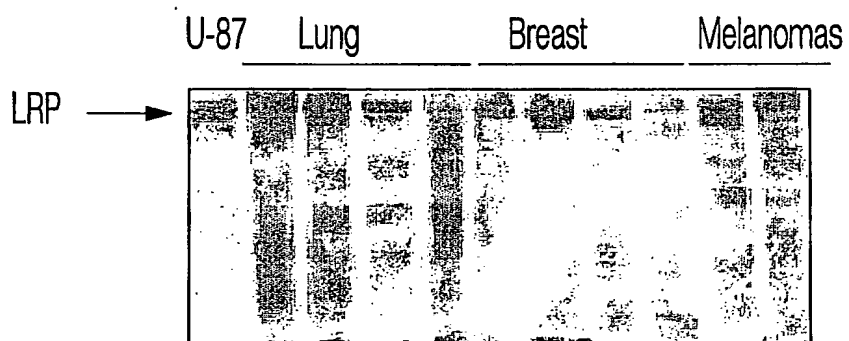
15/28

LRP expression in brain tumors

A. Primary brain tumors



B. Brain metastasis from

FIG-16

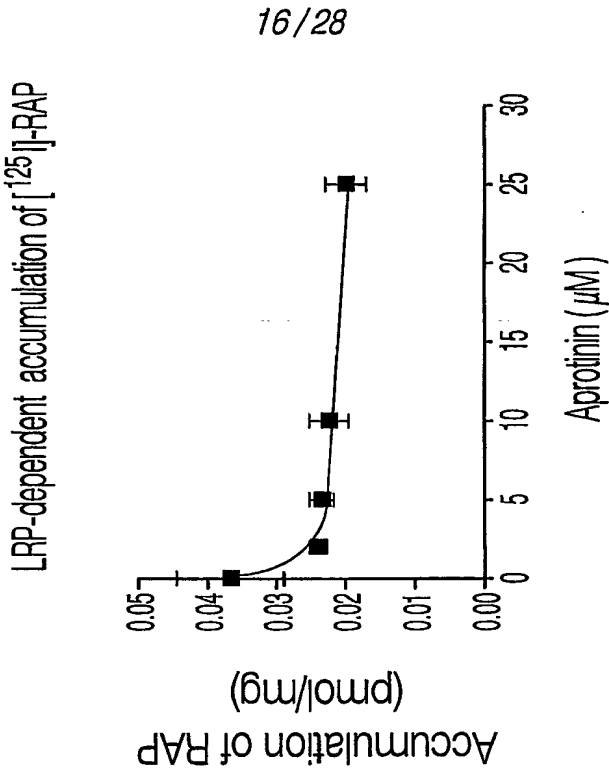


Fig-17B

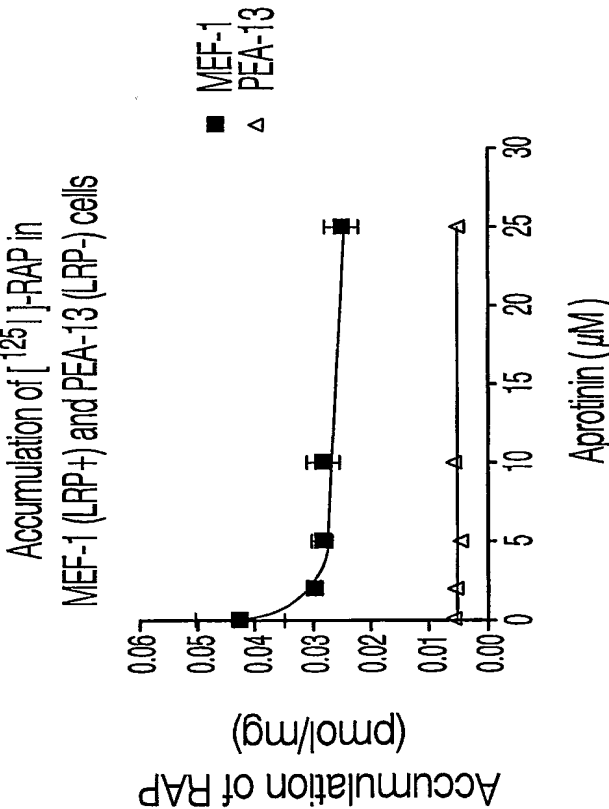
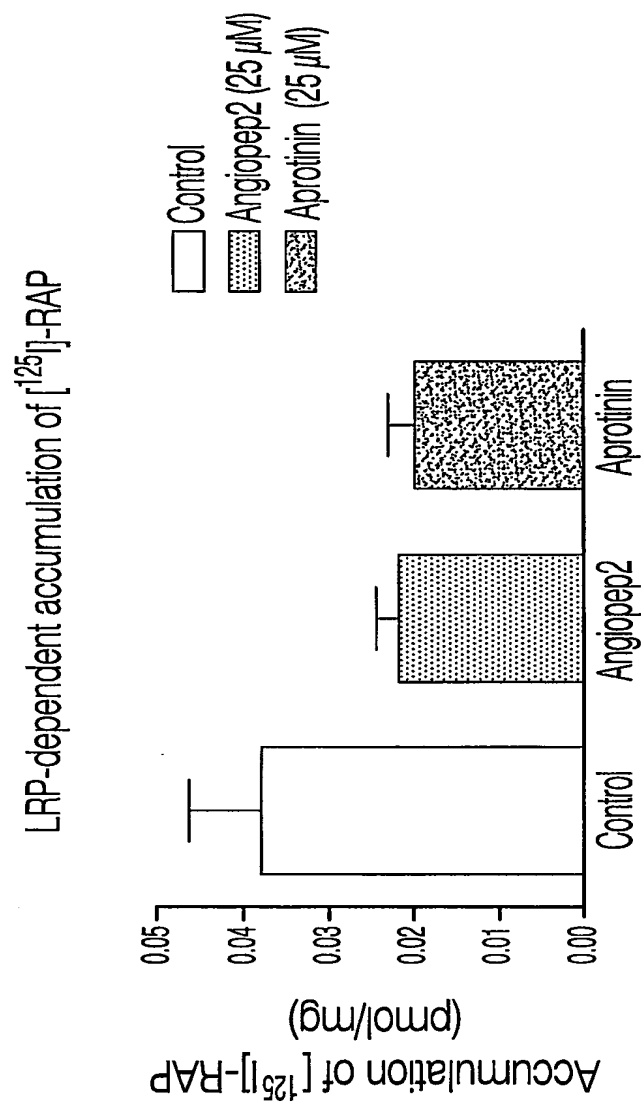


Fig-17A

17/28

**Fig-1B**

18/28

Blood kinetic of TxIAr2 (3:1) (DMSO)

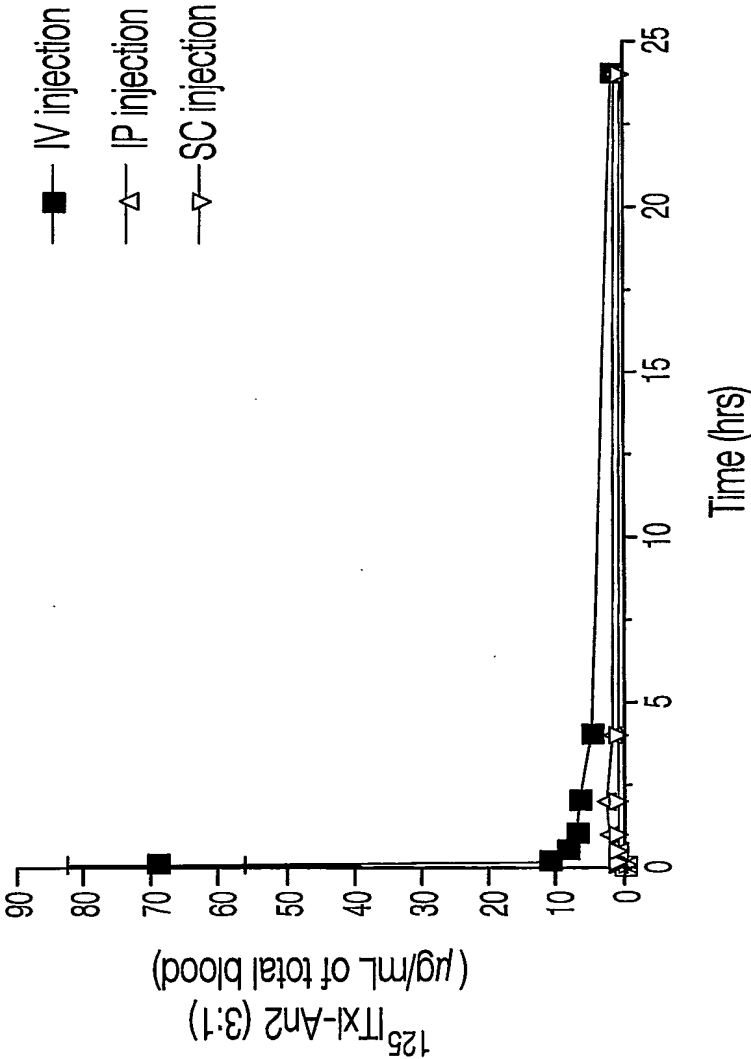
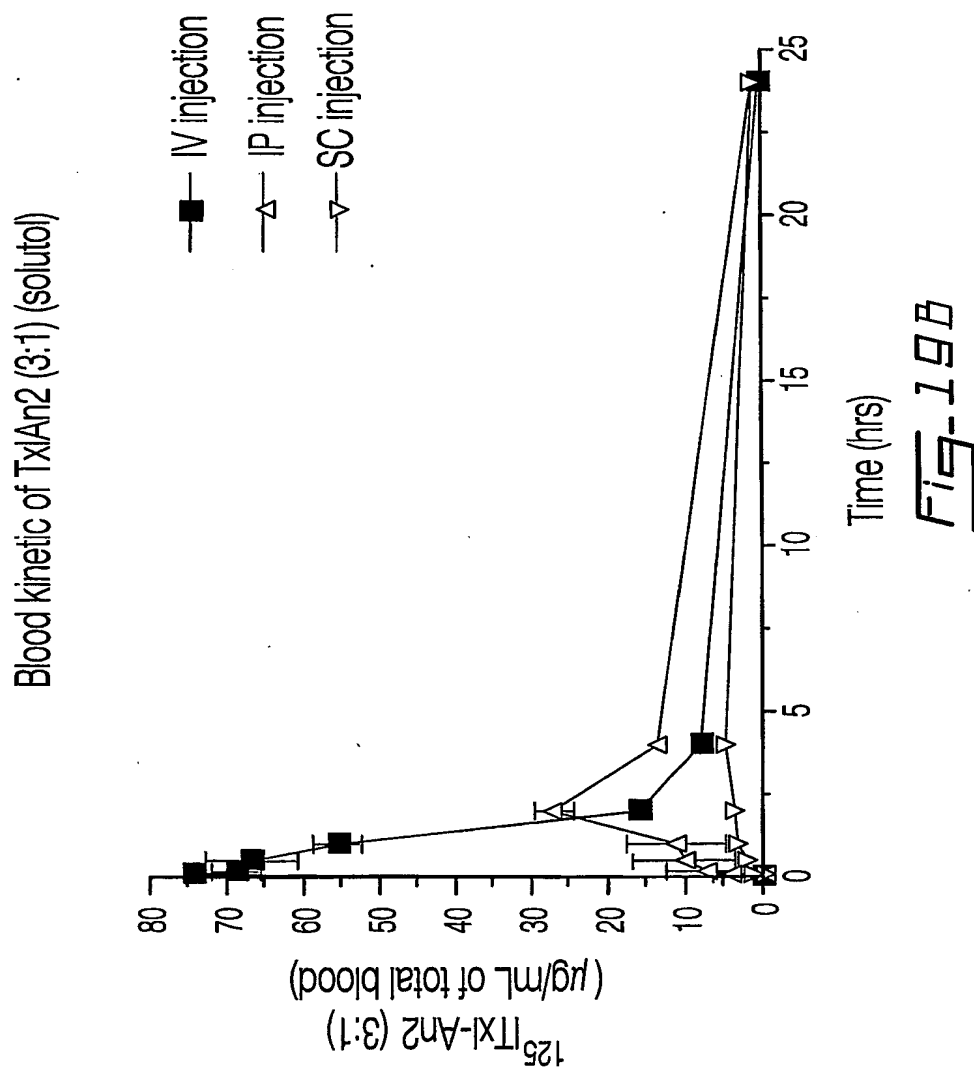
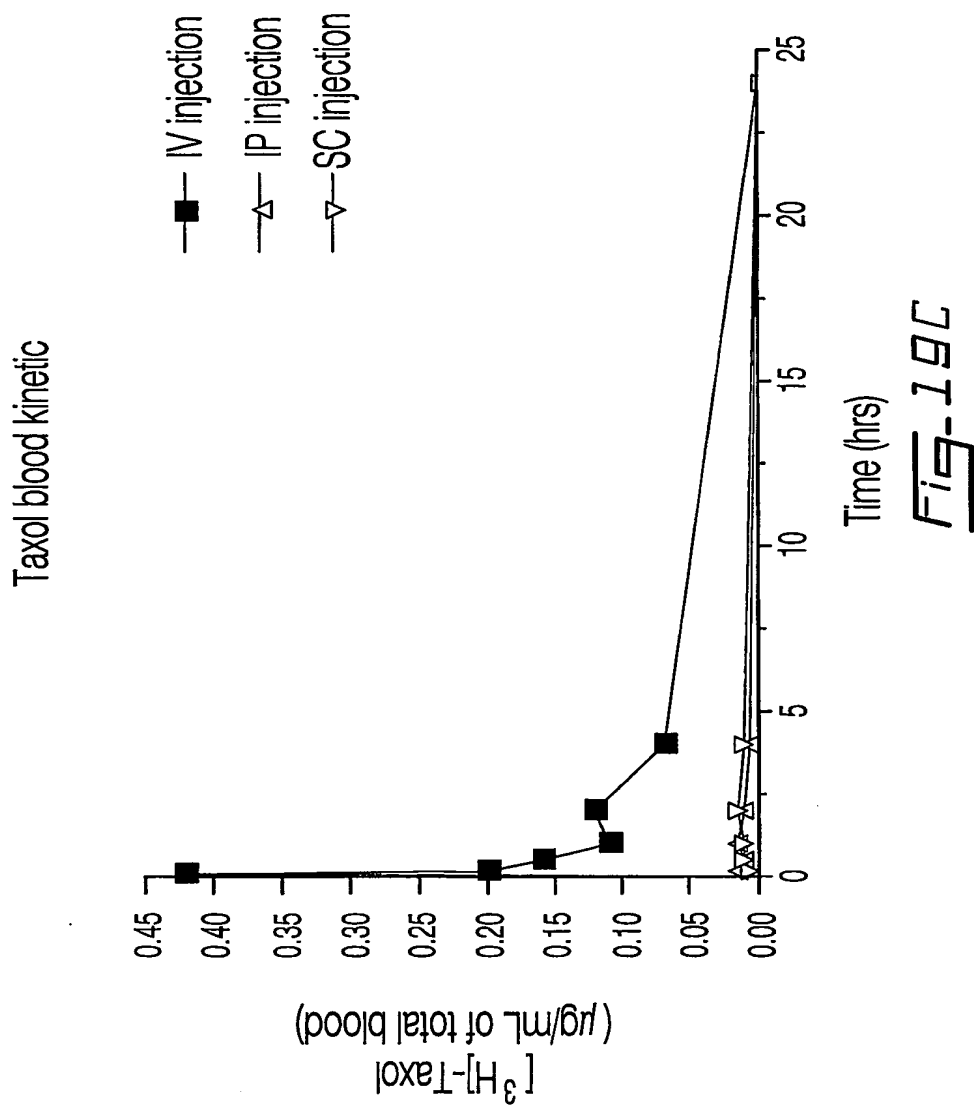


Fig-19A

19/28



20/28



21/28

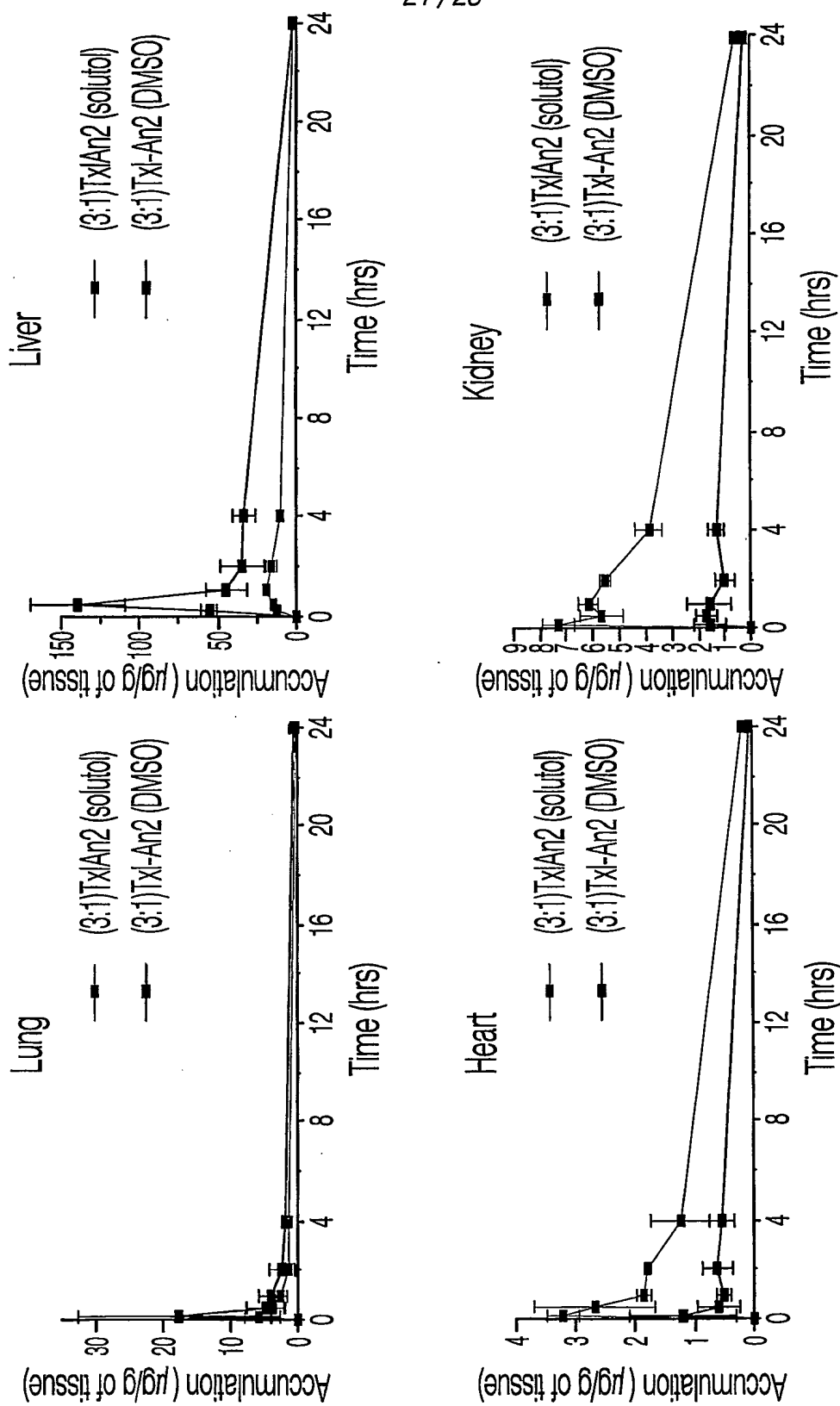


FIG-20A

22/28

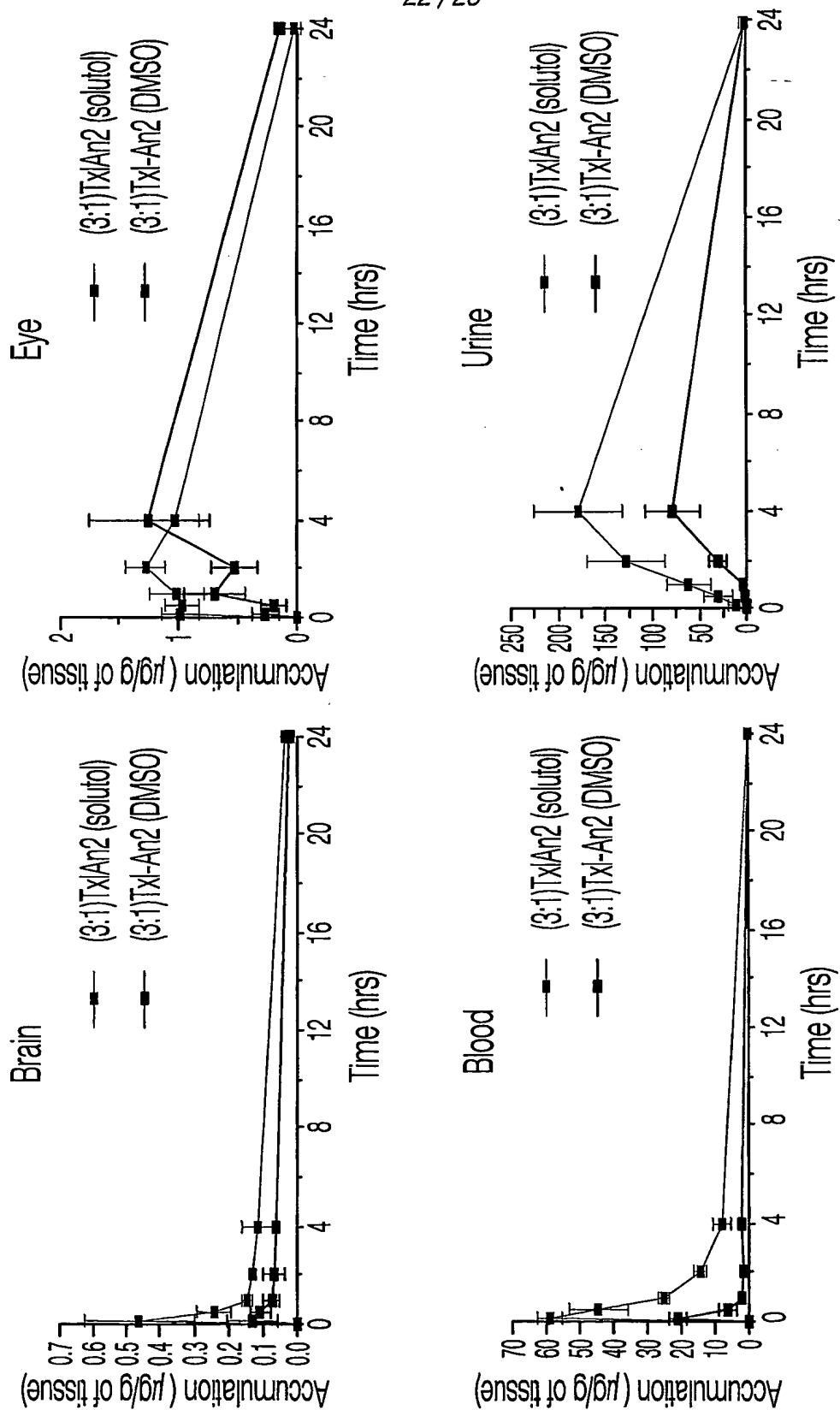


Fig-20B

23/28

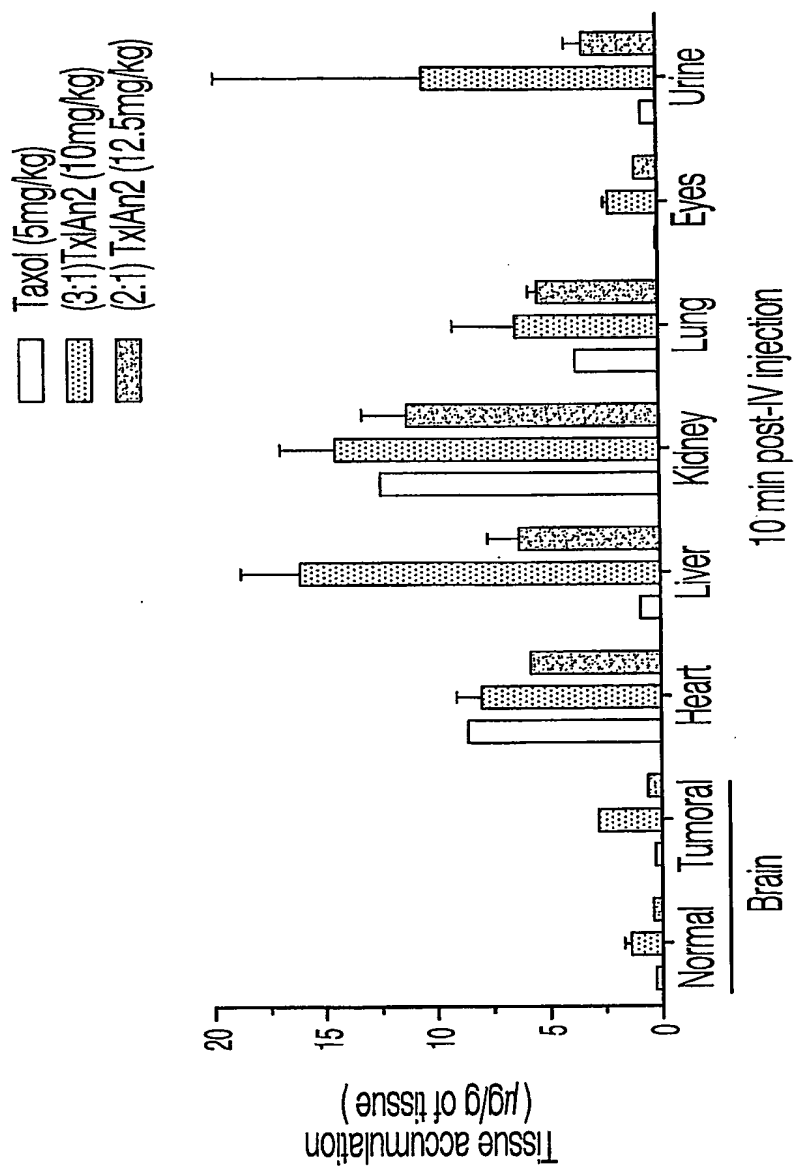


Fig-21A

24/28

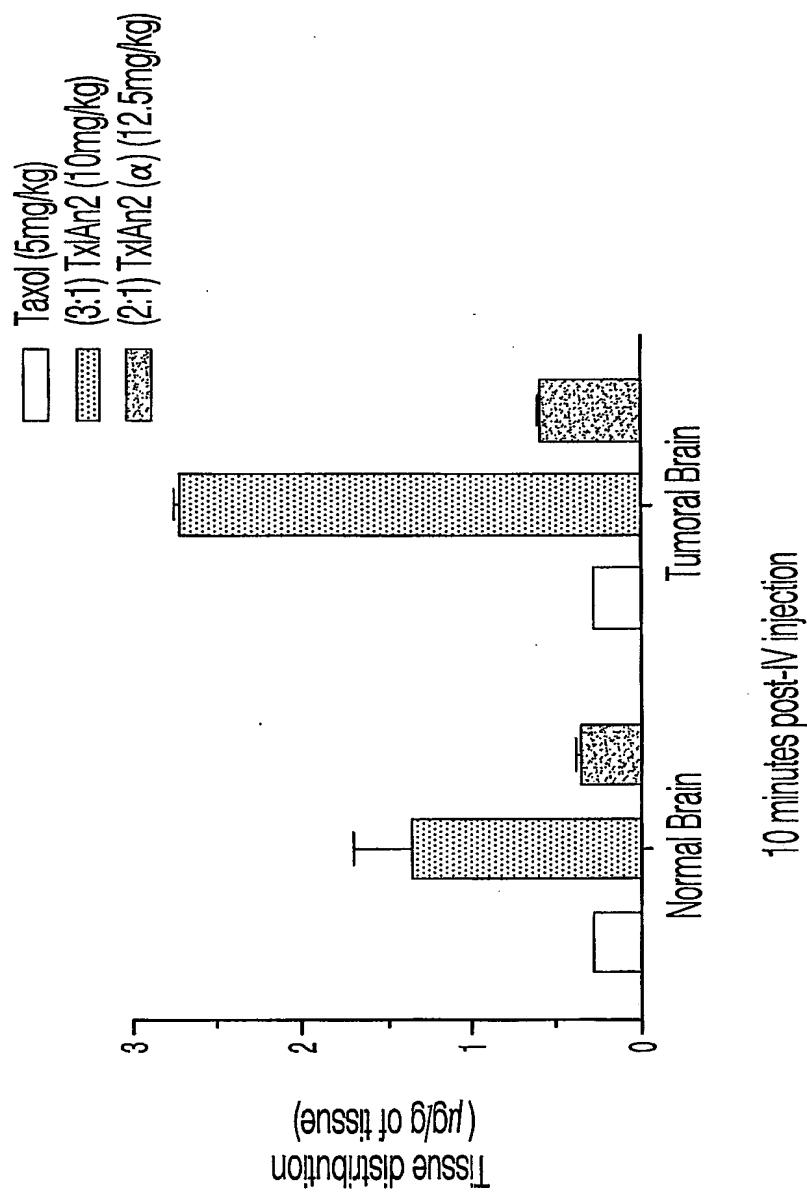


Fig-21B

25/28

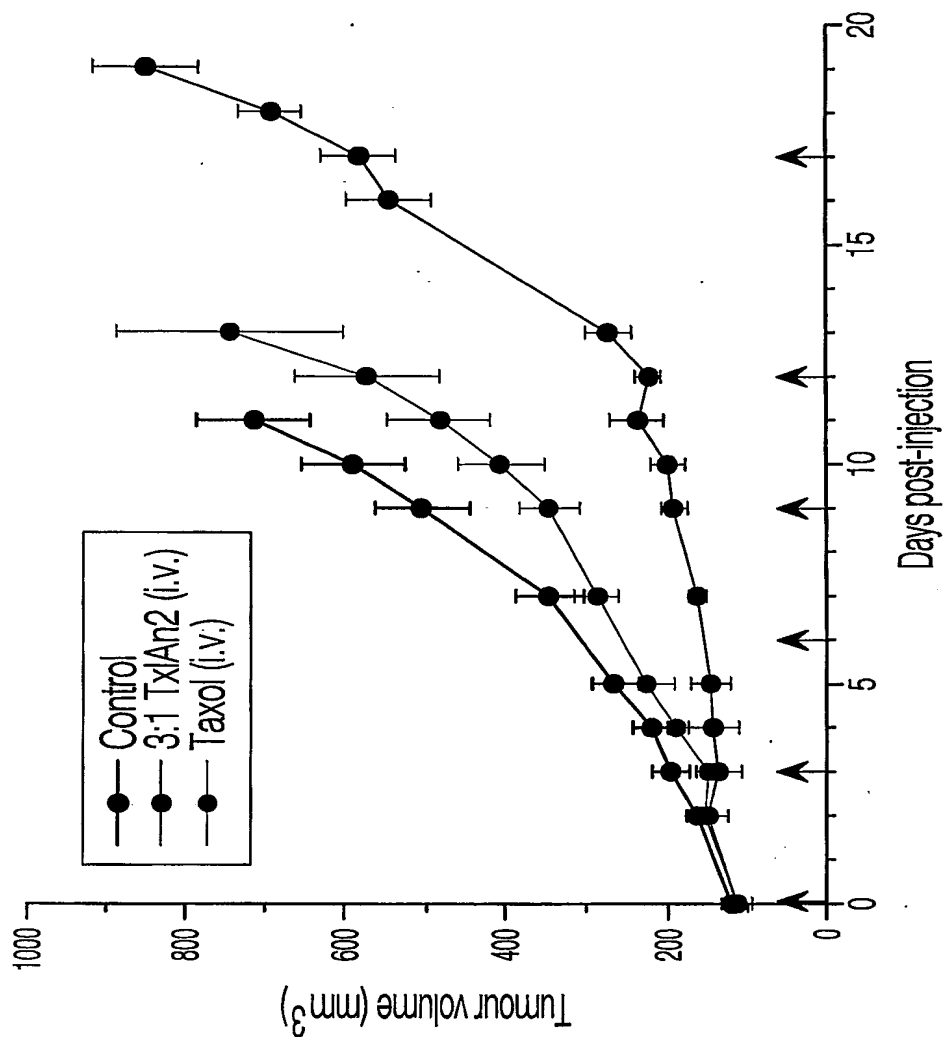


Fig-22A

26/28

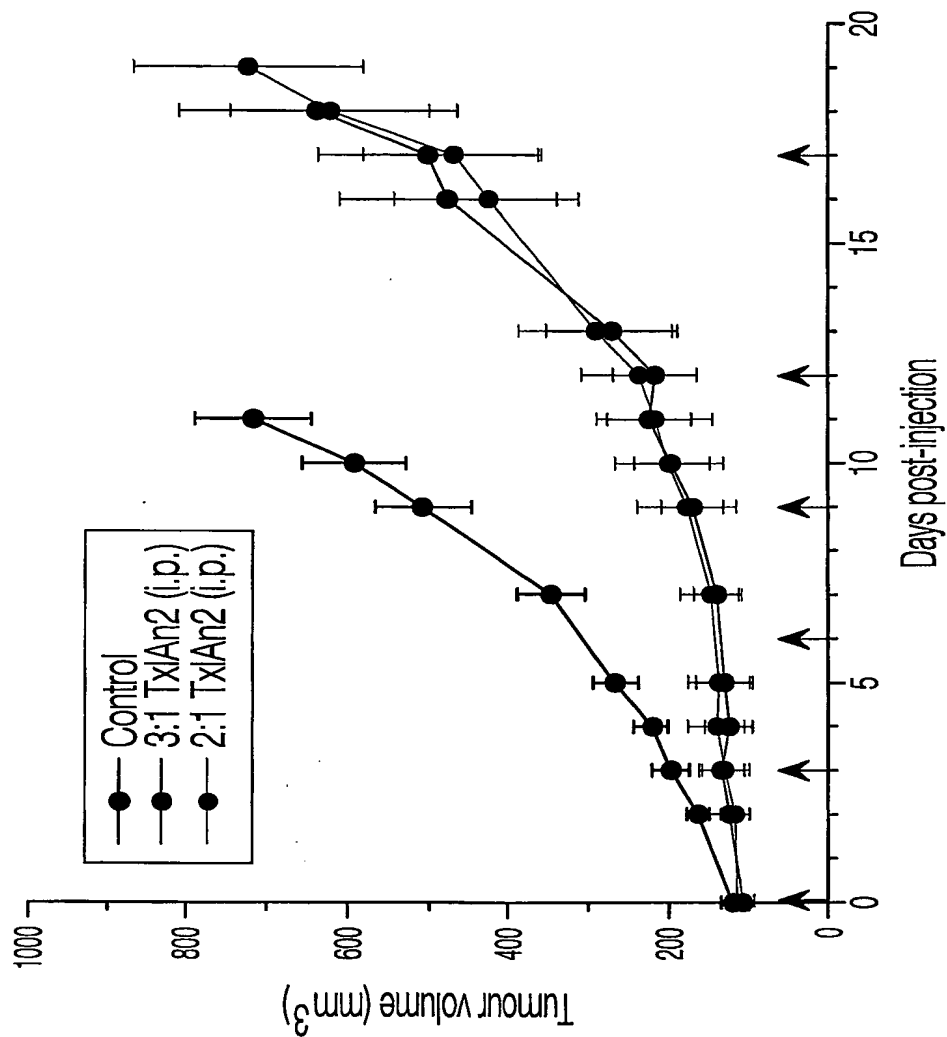


FIG-22B

27/28

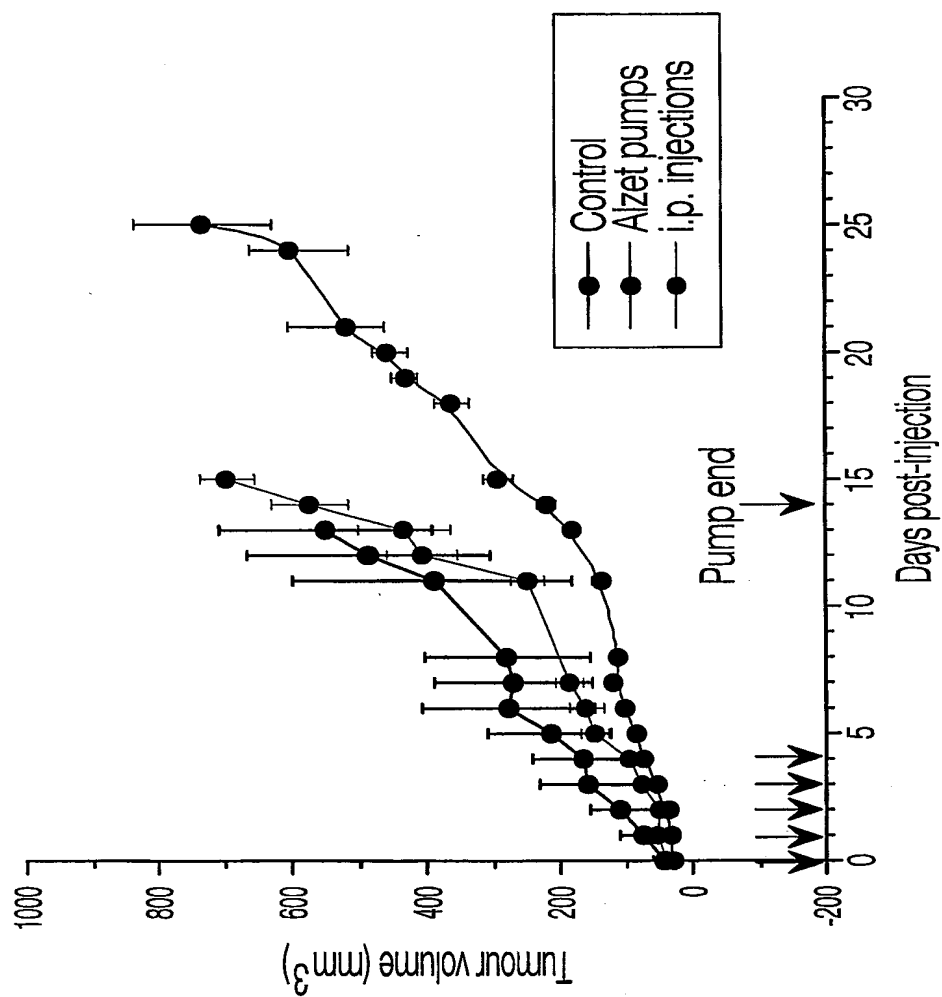


Fig-23A

28/28

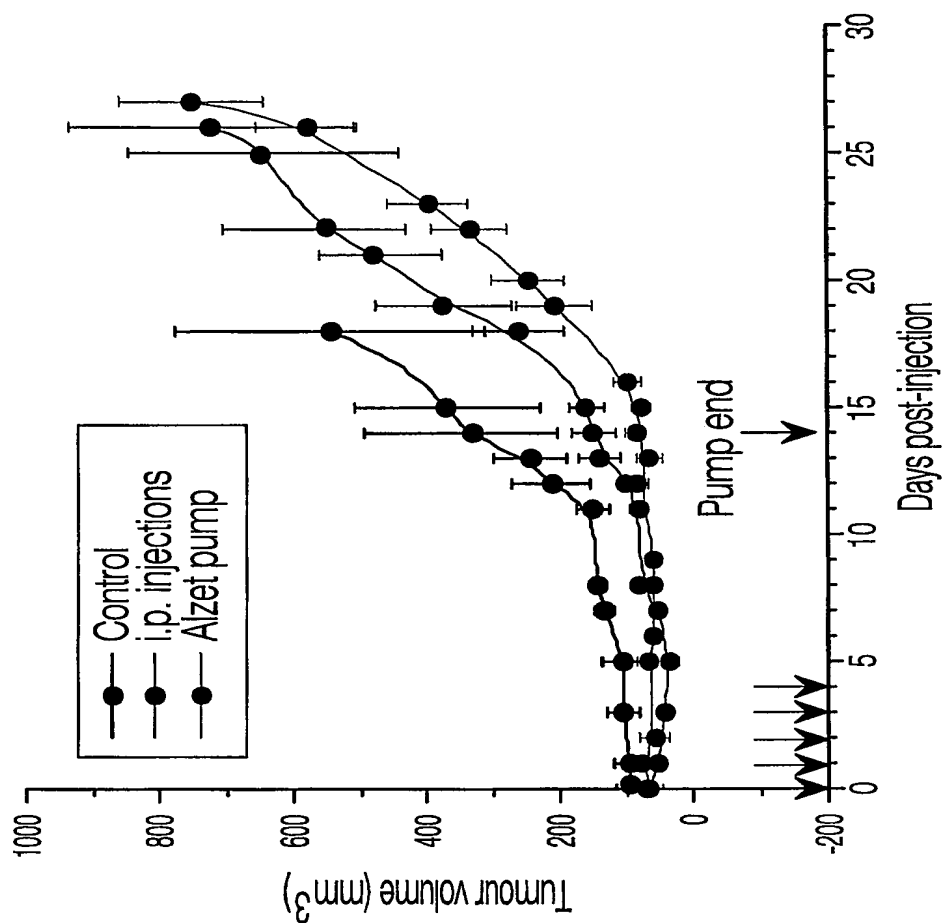


Fig-23B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2006/001165

A. CLASSIFICATION OF SUBJECT MATTER

IPC: **A61K 47/48** (2006.01) , **A61K 31/475** (2006.01) , **A61K 31/196** (2006.01) , **A61K 31/704** (2006.01) ,
A61K 47/42 (2006.01) , **A61P 35/00** (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K 47/48 (2006.01) , **A61K 31/475** (2006.01) , **A61K 31/196** (2006.01) , **A61K 31/704** (2006.01) ,
A61K 47/42 (2006.01) , **A61P 35/00** (2006.01)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic database(s) consulted during the international search (name of database(s) and, where practicable, search terms used)

Canadian Patent Database, United States Patent Database, Espacenet, Delphion, GenomeQuest, Pubmed, Scirus, Scopus, Google
(Keywords: Aprotinin, Angiopep, amyloid B, conjugate, carrier, blood-brain barrier, neurological diseases, label, drug, Taxol,
pharmacokinetics, uptake, antitumor, metastasis, brain and related terms)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CA2525236 (ZANKEL et al.) January 13, 2005	1, 2, 5, 6, 9, 11-20, 22-39, 41-44, 48-63, 65-68, 70-73, 75-89 and 91
X	WO2004060403 (BÉLIVEAU et al.) July 22, 2004 (cited in the application)	1, 2, 5, 6, 9-12, 14-20, 22, 24-34, 36-39, 41-44, 47, 48, 50, 52-62, 65-68, 70-76, 78 and 84-89
Y		13, 21, 23, 35, 40, 45, 46, 49, 51, 63, 64, 69, 77, 79-83 and 90-92
X	WO2003009815 (BÉLIVEAU et al.) February 6, 2003	1, 2, 5, 6, 9, 11-20, 22-34, 36-39, 41-44, 48-62, 65-68, 70-73, 75-89 and 91
X	CA2283474 (PANET et al.) September 11, 1998	1, 2, 5, 6, 9, 11-13, 18-20, 22, 23, 26-35, 39, 41-44, 47-49, 52-61, 68, 70-74 and 77-79

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier application or patent but published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search 30 October 2006 (30-10-2006)	Date of mailing of the international search report 09 November 2006 (09.11.2006)
Name and mailing address of the ISA/CA Canadian Intellectual Property Office Place du Portage I, C114 - 1st Floor, Box PCT 50 Victoria Street Gatineau, Quebec K1A 0C9 Facsimile No.: 001(819)953-2476	Authorized officer Wesley Sharman 819-934-2326

INTERNATIONAL SEARCH REPORTInternational application No.
PCT/CA2006/001165**Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of the first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons :

1. ☒ Claim Nos. : 54-92

because they relate to subject matter not required to be searched by this Authority, namely :

Although claims 54-92 are directed to methods of medical treatment of the human or animal body, a search has been carried out on the alleged effects of the defined conjugates.

2. ☐ Claim Nos. :

because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically :

3. ☐ Claim Nos. :

because they are dependant claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows :

See extra sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☒ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claim Nos. :
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim Nos. :

Remark on Protest ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2006/001165

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DE19953696 (CHERKAKSY) May 10, 2001	1, 2, 5, 6, 9, 28, 31-34, 52-58, 68 and 70
X	KIERNAN et al. "Fluorescent-labelled aprotinin: A new reagent for the histochemical detection of acid mucosubstances" <i>Histochemie</i> , 1973, 34, 77-84.	1, 2 and 6
Y	RAMAKRISHNAN "The role of P-glycoprotein in the blood-brain barrier" <i>Einstein Quart. J. Biol. Med.</i> , 2003, 19, 160-165.	13, 21, 23, 35, 40, 45, 46, 49, 51, 63, 64, 69, 77, 79-83 and 90-92
Y	SCHINKEL "P-glycoprotein, a gatekeeper in the blood-brain barrier" <i>Advanced Drug Delivery Reviews</i> , 1999, 36, 179-194.	13, 21, 23, 35, 40, 45, 46, 49, 51, 63, 64, 69, 77, 79-83 and 90-92
Y	FROMM "P-glycoprotein: A defense mechanism limiting oral bioavailability and CNS accumulation of drugs" <i>International Journal of Clinical Pharmacology and Therapeutics</i> , 2000, 38(2), 69-74.	13, 21, 23, 35, 40, 45, 46, 49, 51, 63, 64, 69, 77, 79-83 and 90-92
E	WO2006086870 (BÉLIVEAU et al.) August 24, 2006	1, 2, 5, 6, 9-12, 14-20, 22, 24-34, 36-39, 41-44, 47, 48, 50, 52-62, 65-68, 70-76, 78 and 84-89
A	LARIONOVA et al. "Carbohydrate-containing derivatives of the trypsin-kallikrein inhibitor aprotinin from Bovine Organs II. Inhibitor coupled to the (carboxymethyl)dextran derivatives of D-galactose" <i>Biol. Chem. Hoppe-Seyler</i> , 1985, 366, 743-748.	

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/CA2006/001165

Patent Document Cited in Search Report	Publication Date	Patent Family Member(s)	Publication Date
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DE19953696	10-05-2001	none	
WO2006086870	24-08-2006	US2006189515 A1	24-08-2006

Continuation of Box III.

The claims of the present international application are directed to a plurality of alleged inventions as follows:

Group A: Claims 1-8 and 9-51 (in part) are directed towards improved formulations of conjugates comprising a carrier selected from the group consisting of aprotinin, a biologically active aprotinin fragment, Angiopep-1, Angiopep-2 and biologically active analogs, derivatives and fragments thereof and a label or small molecule drug able to reduce the growth of a cell and the use of such improved formulations.

Group B: Claims 9-51 (in part) and 52-92 are directed towards the use of a carrier selected from the group consisting of aprotinin, a biologically active aprotinin fragment, Angiopep-1, Angiopep-2 and biologically active analogs, derivatives and fragments thereof in promoting the accumulation inside a cell and altering the pharmacokinetics of a compound selected from a label, a protein, a peptide and a small molecule drug. This promotes the use of these compounds in reducing the growth of a cell and in the treatment of cancer and metastatic cancer.

As such, the claims on file do not comply with the requirements of Rule 13 (PCT).

Conjugates comprising aprotinin, a biologically active aprotinin fragment, Angiopep-1, Angiopep-2 and biologically active analogs, derivatives and fragments thereof with a compound selected from a label, a protein, a peptide and a small molecule drug are known. As a result, there is no single linking inventive concept between allegedly new formulations comprising these conjugates (in particular formulations comprising a solubilizer such as Solutol® HS-15) (see in particular pages 52-56 of the current description) and allegedly new uses of these conjugates. Even though the allegedly new formulations may have these same allegedly new uses, the potential linking technical feature (the conjugates themselves) is known and thus, does not provide any unifying inventive concept.